Characterization of inflammation in COPD : clinical and experimental approach

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Characterization of inflammation in COPD

Clinical and experimental approach

Juanita Henriëtte Jacqueline Vernooy

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The Graduate School

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Characterization of inflammation in COPD

Clinical and experimental approach

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*Denken is makkelijk, maar doen is moeilijk,
en om je gedachten om te zetten in daden
is het moeilijkste ter wereld*

Johann Wolfgang von Goethe
Schrijver, filosoof en wetenschapper

Ter nagedachtenis aan Pap
Voor Mam
Voor Alex

Table of Contents

CHAPTER 1	General introduction	9
CHAPTER 2	Aims and outline of the thesis	37
CHAPTER 3	Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble TNF-receptors are increased in sputum	39
CHAPTER 4	Local and systemic acute-phase response in COPD	55
CHAPTER 5	Leptin as local inflammatory marker in COPD	67
CHAPTER 6	Increased activity of matrix metalloproteinase (MMP)-8 and MMP-9 in induced sputum from patients with COPD	73
CHAPTER 7	Intratracheal instillation of lipopolysaccharide in mice induces apoptosis in bronchial epithelial cells: no role for TNF- α and infiltrating neutrophils	85
CHAPTER 8	Rapid expression of acute-phase reactants in lung after local LPS exposure in mice	99
CHAPTER 9	Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology	113
CHAPTER 10	Discussion and future directions	129
CHAPTER 11	Summary	135
CHAPTER 12	Nederlandse samenvatting	139
APPENDICES	Abbreviations	145
	Dankwoord	147
	List of publications	151
	Curriculum vitae	155

CHAPTER 1

General introduction

1. CHRONIC OBSTRUCTIVE PULMONARY DISEASE

1.1 Prevalence and health care costs

Chronic obstructive pulmonary disease (COPD) is a complex heterogeneous respiratory disease, characterized by the progressive development of airflow limitation that is largely irreversible^{1,2}. Three conditions comprise COPD, namely chronic bronchitis (mucus hypersecretion), chronic bronchiolitis (small airway disease), and emphysema (alveolar destruction)³. The contribution of each component to airflow limitation, pathophysiology, and clinical symptoms varies between individual patients. COPD represents a major and growing health care problem. To date, COPD is the fourth leading cause of death in the Netherlands, and will be the third leading cause worldwide in 2020, with an expected mortality of 4.7 million persons annually⁴. Reasons for the dramatic increase in COPD prevalence in the next 20 years include a marked increase in cigarette smoking and environmental pollution in developing countries, along with reduced mortality from other causes, such as cardiovascular diseases in industrialized countries and infectious diseases in developing countries⁵. In the Netherlands, approximately 73 per 1000 persons are diagnosed with COPD. The clinical characteristics of COPD (a rapid decline in lung function or persistently decreased lung function) are observed in 20% of the general adult population in the Netherlands⁶. Moreover, the total COPD-related medical costs are a major burden for the Dutch health care system. The direct costs of COPD represent 1.3% of the Dutch health care budget and are expected to increase by 60% in the near future, mostly due to aging of the population⁷.

1.2 Risk factors

Risk factors for COPD are thought to include both host factors and environmental exposures, and the disease is suggested to arise from an interaction between these two types of factors⁴. The best-documented host factor for the development of COPD is hereditary severe α_1 -antitrypsin (α_1 -AT) deficiency, as shown by a proteinase inhibitor phenotype (PiZZ) with α_1 -AT levels below 10% of normal values⁸. Lack of this serum protein, which can be inherited as an autosomal recessive trait, can be associated with accelerated decline of development of emphysema (not chronic airway diseases) in non-smokers, and a markedly accelerated rate in smokers. However, less than 1% of patients with COPD has severe α_1 -AT deficiency⁹. In addition, many other genetic variants of α_1 -AT that are

associated with lower-than-normal serum levels of this proteinase inhibitor have not been clearly associated with an increased risk for COPD⁸.

Cigarette smoking is widely recognized as the most important environmental risk factor leading to the development of COPD. Age at starting to smoke, total pack-years smoked, and current smoking status are predictive of COPD mortality¹. The presence of harmful compounds in inhaled cigarette smoke is considered to be the starting point of the pathogenetic pathway in COPD. In industrialized countries, smoking accounts for as much as 80 to 85% of cases of COPD¹. There is a considerable variation among individuals in susceptibility to cigarette smoke, as only 10-20% of smokers will develop clinically symptomatic COPD, hereby suggesting the importance of undefined genetic factors in the development of COPD¹⁰. In these so-called susceptible smokers, the rate of decline of lung function, measured as the forced expiratory volume in 1 second (FEV₁), shows a strong relationship with the total pack-years smoked¹. As shown in Figure 1¹¹, smoking cessation is associated with a reduction in the accelerated rate of decline in FEV₁. This reduction was shown to be the largest for younger subjects and decreased linearly with age¹². Recent data from the Lung Health Study demonstrated that the subsequent rate of decline of FEV₁ among sustained quitters was half the rate among continuing smokers in both male and female^{13,14}. In addition, smoking cessation was found to be associated with significant reductions in cardiovascular morbidity and mortality¹⁵. Therefore, quitting smoking is an important therapeutic intervention both to reduce symptoms in patients and, even more important, to prevent the development of COPD. However, the slope of lung function decline remains increased in susceptible ex-smokers when compared to non-smokers *cq.* non-susceptible smokers. As a result, many susceptible ex-smokers, who are currently not symptomatic, will still develop COPD in the future.

Figure 1. Annual decline in lung function is accelerated in patients with COPD¹¹.

Occupational exposures to organic dust, such as grain dust, swine dust, and cotton dust, have also been associated with development and/or progression of COPD when the exposures are sufficiently intense or prolonged. Such exposures can both cause COPD independently of cigarette smoking and increase the risk in the presence of concurrent cigarette smoking¹⁶. For example, follow-up studies have shown that long-term exposure to grain dust or swine dust causes a profound pulmonary inflammatory response related to respiratory symptoms and accelerated longitudinal decline in lung function¹⁷, and thus irreversible and progressive lung pathology. Exposure-response studies have shown that these respiratory symptoms and declines in lung function are dose-dependent^{18,19}.

Furthermore, exposures to sufficient levels of anorganic dusts is suggested to be a risk factor to develop COPD. For example, cumulative respiratory exposure to coal or gold dust is associated with loss of lung function in miners²⁰. In addition, several cross-sectional studies consistently found a higher prevalence of symptoms of hypersecretion, breathlessness, or diagnosis of chronic bronchitis, emphysema, or COPD in areas with higher urban air pollution (sulfur dioxide, ozone, and particulates)²¹.

Next to the factors mentioned above, a number of other factors are likely associated with development of COPD. These include passive smoke exposure, respiratory virus infection, exposure to occupational chemicals (like cadmium), indoor cooking, socioeconomic factors, nutrition, alcohol ingestion, age, gender, mucus hypersecretion, and airway hyperresponsiveness (reviewed in detail by Buist and Vollmer²²).

1.3 Pathogenesis

There is strong evidence that COPD is associated with a chronic inflammatory response throughout the airways, lung parenchyma, and pulmonary vasculature, which is initiated by the inhalation of noxious particles and gases such as those present in cigarette smoke⁴. The lung has innate defense mechanisms and a considerable capacity to repair itself²³, but these mechanisms may be affected by the genetic background or (repeated) exposure to environmental risk factors. The chronic inflammatory response is characterized by cellular inflammatory infiltrates as well as production of inflammatory mediators, proteinases, and reactive nitrogen and oxygen species. The latter molecules may be produced by inflammatory cells or they may arise from environmental factors. The intensity and characteristics (both cellular and molecular) of the inflammation vary as the disease progresses. The pathologic changes characteristic for COPD are eventually caused either directly by inhaled noxious particles, or, indirectly by the action of inflammatory mediators⁴.

Inflammatory cells

Many studies reveal a correlation between the number of inflammatory cells of various types in the lung and the severity of COPD²⁴⁻²⁹. However, the types of inflammatory cells vary in the different compartments of the lung. Neutrophils are short-lived, transient cells that are recruited directly from peripheral blood to the

airways. A marked increase in neutrophils in sputum and bronchoalveolar-lavage (BAL) fluid, reflecting neutrophilia in the airway compartment³⁰, was demonstrated in COPD patients compared to controls^{26,27}. The extent of neutrophilia correlated with the degree of airflow limitation^{27,28,31}. In addition, elevated levels of neutrophil granule proteins, such as myeloperoxidase (MPO) and human neutrophil lipocalin (HNL), were demonstrated in induced sputum and BAL fluid from severe COPD patients and smoking subjects with subclinical emphysema, respectively, indicating activation of recruited neutrophils^{32,33}. In contrast, neutrophils are little increased in airway and lung parenchyma tissue sections, which may reflect their rapid transit through these parts of the lung. As neutrophils have both a strong potential to produce oxygen radicals and to secrete several proteinases, including neutrophil elastase (NE), cathepsins, and matrix metalloproteinases (MMPs)³⁴⁻³⁶, they may significantly contribute to parenchymal destruction and chronic mucus hypersecretion.

Increased numbers of macrophages are present in the large and small airways and lung parenchyma of COPD patients, as observed by histopathology, in BAL fluid, bronchial biopsies, and induced sputum studies^{26-29,37-39}. Macrophages are long-lived cells derived from peripheral blood monocytes, and are 5 to 10 times more numerous than other inflammatory cells. Macrophages are thought to play a critical orchestrating role in both the persistence of the chronic inflammatory response and the proteolytic matrix degradation in COPD. Activated macrophages release for example neutrophil-chemotactic factors, such as TNF- α , leukotriene B₄ and interleukin-8, hereby promoting neutrophil recruitment⁵. In addition, macrophages secrete a spectrum of potent proteolytic enzymes, including MMP-1, MMP-9, and MMP-12, that break down connective tissue in the lung parenchyma and stimulate mucus secretion³⁶.

Other inflammatory cell types noted in the lung of COPD patients include T lymphocytes, predominantly CD8+ (cytotoxic) T cells, and eosinophils. Histopathology and bronchial biopsy studies show an increase in cytotoxic T cells throughout the whole lung^{25,29,40-42}. Although their role in COPD inflammation is not yet understood, cytotoxic T-cells may be involved in the apoptosis and destruction of alveolar-wall epithelial cells through the release of perforin, granzyme B, and TNF- α ⁵. The reports on the presence and role of eosinophils in COPD are contradictory. However, most studies agree that airway eosinophils are not prominent in COPD except during acute exacerbations or in patients with concomitant asthma.

Next to inflammatory cells, resident cells within the lung are likely to contribute to inflammation in COPD. The epithelium is a target for factors released by infiltrating inflammatory cells and serves as a major effector of inflammation⁴³. On encountering such agents, airway and alveolar epithelial cells can produce a spectrum of proinflammatory mediators, including cytokines, chemokines, acute-phase proteins, lipid mediators, and reactive nitrogen and oxygen species, which can further effect pathophysiological alterations like mucus hypersecretion and influx of inflammatory cells⁴⁴. The endothelium plays also a significant role in inflammatory processes. In COPD, pulmonary endothelium

demonstrated increased expression of adhesion molecules like E-selectin and ICAM-1, which serve as anchors for circulating inflammatory cells to transmigrate into the lung tissue^{45,46}. To date, the actual role of epithelial cells and endothelial cells in the chronic inflammation in COPD has not been thoroughly studied.

Inflammatory mediators

Migration and activation of inflammatory cells is regulated by cytokines and chemokines secreted by a variety of structural cells, such as epithelial, endothelial, smooth muscle, and fibroblasts, as well as by activated inflammatory cells. In COPD, increased levels of cytokines (TNF- α) and chemokines (IL-8, LTB₄, MCP-1, MIP-1 β , GRO- α) have been demonstrated in induced sputum and/or BAL fluid as compared to control subjects^{28,47-49}. In addition, elevated expression of IL-8, MIP-1 α , MCP-1, IP-10 and TGF- β and/or their mRNA has been observed in bronchiolar epithelium, and of the chemokine receptor CCR-2 in macrophages from smokers with COPD compared with those without COPD^{31,50-53}. Yet limited information is available concerning the specific role of these inflammatory mediators involved in the pathogenesis of COPD.

Proteinase-antiproteinase imbalance

Neutrophils and macrophages can potentially produce large quantities of proteinases that play a significant role in restoring tissue homeostasis after inflammatory processes. In COPD, an imbalance of proteinases and antiproteinases is thought to contribute to destruction and remodelling of the extracellular matrix (ECM), the critical contributor to the integrity of the lung, resulting in loss of recoil and eventually emphysema. This imbalance may involve increased production or activity of proteinases, or inactivity or reduced production of antiproteinases, or both. However, the cell types and proteinases responsible for ECM destruction and the ECM components that are targets of the proteinases have not yet been identified⁵⁴. Concerning the proteinases involved, the attention has been primarily focussed on neutrophil elastase based on the increased prevalence of emphysema in patients with congenital deficiency of α_1 -AT, an endogenous inhibitor of neutrophil elastase⁸. However, a large majority of patients with α_1 -AT deficiency do not develop emphysema and success of clinical trials with α_1 -AT supplementation therapy in both α_1 -AT deficient patients and patients with primary COPD is limited⁵⁵. The α_1 -AT/neutrophil elastase concept is further challenged by histological and biochemical studies. First, no apparent association between accumulation of neutrophils and parenchymal destruction was found in COPD patients⁵⁶. Second, structural support of cells in the lung is primarily dependent on interstitial collagens (predominantly collagen type I and III), which are highly resistant to proteolytic degradation, and can only be cleft by specific collagenases⁵⁷. Therefore, additional proteinases may be involved in COPD caused by inhalational exposures, such as cathepsins and matrix metalloproteinases (MMPs). Cathepsins comprise a heterogenous family of lysosomal cysteine proteases with enormous disruptive potential⁵⁸. Elevated levels of cathepsin L and G have been demonstrated in BAL fluid or sputum sol phase, respectively, of

patients with COPD^{59,60}. MMPs comprise a large family of structurally related zinc metallo-endopeptidases with different substrate specificities, collectively able to degrade most, if not all, protein constituents of the extracellular matrix^{57,61}. Importantly, MMPs are secreted in latent form and activated by removal of the amino-terminal propeptide-sequence. The net MMP activity *in vivo* is therefore dependent on the balance between activated MMP pro-enzymes and the natural Tissue Inhibitors of Metalloproteinases (TIMPs)^{57,61}. Recent studies in man support that members of the MMP family are critically involved in the development and progression of emphysema. Increased concentrations of MMPs were found in induced sputum (MMP-9) and BAL fluid (MMP-2, MMP-8, and MMP-9) of COPD patients^{33,62,63}. In addition, increased levels of TIMP-1 were measured in COPD patients versus non-smokers^{62,64}. In emphysematous lung tissue, increased levels of both protein and mRNA for MMP-1, MMP-2, MMP-8 and MMP-9 were demonstrated^{63,65,66}. However, studies investigating MMP activity levels are limited and a causal relationship between MMP activity and tissue destruction has not yet been demonstrated.

Oxidative stress

Oxidant/antioxidant imbalance, in favor of oxidants, is another important feature in COPD. Markers of oxidative stress have been found in the epithelial lining fluid, breath, and urine of patients with COPD. For example, hydrogen peroxide (H_2O_2) and nitric oxide (NO), which are direct measures of oxidants generated by cigarette smoking or released from inflammatory leukocytes and epithelial cells, are increased in the breath during exacerbations of COPD^{67,68}. Oxidative stress may contribute to COPD in numerous ways⁶⁹. First, oxidants can react with, and damage, a variety of biological molecules (proteins, lipids, nucleic acids), leading to cell dysfunction or death, as well as damage to the lung extracellular matrix. Second, oxidative stress contributes to the proteinase-antiproteinase imbalance both by inactivating antiproteinases (such as α_1 -antitrypsin and SLPI) and by activating proteinases (such as MMPs). Third, oxidants promote inflammation, for example by activating the transcription factor NF- κ B that orchestrates the expression of multiple inflammatory genes like IL-8 and TNF- α thought to be important in COPD. Finally, oxidative stress may contribute to reversible airway narrowing.

1.4 Pathology

Pathological changes characteristic of COPD are found in the central airways, peripheral airways, lung parenchyma, and pulmonary vasculature⁴.

Central airways

The chronic inflammation in the central airways (trachea, bronchi, and bronchioles >2-4 mm) of patients with chronic bronchitis is associated with an increase in the number (metaplasia) of epithelial goblet and squamous cells and mucus hypersecretion^{1,69}. In addition, association with dysfunction, damage, and/or loss of cilia; enlarged submucosal mucus-secreting glands; an increase in the amount

of smooth muscle and connective tissue in the airway wall; degeneration of the airway cartilage was found. The various pathological changes in the central airways are responsible for the symptoms of chronic cough and sputum production, which identify people at risk for COPD and may continue to be present throughout the course of the disease.

Peripheral airways

The early decline in lung function in COPD is correlated to the pathologic changes in the peripheral airways (small bronchi and bronchioles < 2 mm), which include exudate of fluid and cells in the airway wall and lumen, goblet and squamous cell metaplasia of the epithelium, edema of the airway mucosa due to inflammation, and excess mucus in the airways due to goblet cell metaplasia⁶⁹. The most characteristic change in the peripheral airways of COPD patients, however, is airway narrowing due to disordered remodeling of the airway wall, which is a result of repeated injury and repair processes initiated by cigarette smoking⁷⁰ and other risk factors. The airway remodelling is characterized by increased collagen deposition and scar tissue formation, and results in significant narrowing of the lumen and fixed airways obstruction⁷¹. Direct measurements of peripheral airway resistance have shown that the structural changes in the airway wall are the most important cause of the increase in peripheral airways resistance in COPD⁷².

Lung parenchyma

The pathologic hallmark of COPD, especially emphysema, is destruction of the lung parenchyma (respiratory bronchioles, alveoli, and pulmonary capillaries). The most common type of parenchymal destruction, defined as non-uniformity in the pattern of respiratory airspace enlargement, in COPD patients is the centrilobular form of emphysema, which involves dilatation and destruction of the respiratory bronchioles, and in advanced disease also of the pulmonary capillary bed⁴. Panacinar (panlobular) emphysema, which extends throughout the acinus¹, is the characteristic lesion seen in α_1 -AT deficiency and involves dilatation and destruction of the alveolar ducts and sacs as well as of the respiratory bronchioles.

Pulmonary vasculature

Pulmonary vascular changes in COPD are characterized by a thickening of the vessel wall that begins early in the natural history of the disease, when lung function is reasonably well maintained⁴. Thickening of the intima is the first structural change⁷³, followed by an increase in vascular smooth muscle and the infiltration of the vessel wall by macrophages and CD8+ T lymphocytes⁴¹. These structural changes are correlated with an increase in pulmonary vascular pressure that develops first with exercise and then at rest. In addition to thickening of the vessel wall, endothelial dysfunction of the pulmonary arteries, which may be caused directly by cigarette smoke products⁷⁴ or indirectly by inflammatory mediators⁴¹, is another early event in the pathology of COPD⁷⁵. Interestingly, recent studies demonstrated decreased expression of VEGF and its receptor (VEGF-2) in emphysematous lung tissue, which was related to increased apoptosis

of alveolar septal cells, both pneumocytes and endothelial cells⁷⁶.

1.5 Pathophysiology

The pathological changes in COPD lead to corresponding physiological abnormalities that usually become evident first on exercise and later also at rest. Expiratory airflow limitation is the physiological hallmark of COPD. Table 1 shows the pathological causes that contribute to airflow limitation in COPD⁶⁹. The airflow limitation characteristic of COPD is usually persistent and typically shows a more rapid progressive deterioration with age than is normal. Other physiological changes in COPD include mucus hypersecretion, ciliary dysfunction, pulmonary hyperinflation, gas exchange abnormalities, pulmonary hypertension, and cor pulmonale, and they usually develop in this order over the course of the disease⁴. In turn, the various physiological abnormalities contribute to the characteristic clinical symptoms of COPD, i.e. chronic cough, sputum production and dyspnea⁶⁹.

Table 1. Pathological causes of expiratory airflow limitation in COPD

Irreversible	Reversible
<ul style="list-style-type: none"> • Fibrosis and narrowing of airways • Loss of elastic recoil due to alveolar destruction • Destruction of alveolar support that maintain patency of small airways 	<ul style="list-style-type: none"> • Accumulation of inflammatory cells in the lung • Accumulation of mucus and plasma exudate in bronchi • Smooth muscle contraction in central and peripheral airways

Adapted from GOLD guidelines⁶⁹.

1.6 Systemic effects

There is increasing evidence of systemic (i.e. extrapulmonary) effects in patients with COPD, namely systemic inflammation and skeletal muscle dysfunction, which have important negative influence on the quality of life⁷⁷⁻⁸⁰. Similarly to the observation in the airways, increased amounts of activated PMN, as demonstrated by enhanced expression of the adhesion molecule CD11b/CD18, were demonstrated in the circulation of COPD patients in a clinical stable condition⁸¹. In line herewith, proof for systemic oxidative stress in smokers and COPD patients was obtained by the observation of a reduction of the total antioxidative capacity, paralleled by enhanced levels of lipid peroxidation products, as indices of overall oxidative stress⁸². Besides activation of PMN, also activation status of circulating lymphocytes has been demonstrated indicated by enhanced expression of cytochrome oxidase⁸³. Several studies investigating inflammatory mediators in COPD demonstrated enhanced circulating concentrations of acute-phase proteins, such as C-reactive protein (CRP), lipopolysaccharide (LPS) binding protein (LBP) and fibrinogen, along with acute-phase cytokines like IL-6 and TNF- α ^{77,78,84-87}. In addition, small but significant increases in plasma levels of soluble receptors for TNF (sTNF-R55 and sTNF-R75) were noted, which are considered to be markers of a pro-inflammatory state because of their shedding from the cell membrane in

response to endogenous TNF- α and other inflammatory mediators.

In addition to systemic inflammation and oxidative stress, profound weight loss is noted in some COPD patients, particularly those with predominant emphysema⁸⁸. The weight loss, particularly depletion of fat free mass, is due to disturbances in energy balance. Leptin is known to regulate the energy balance via a feedback mechanism in which the hypothalamus is involved⁸⁹. Leptin is a hormone produced by the adipose tissue, and circulating leptin levels are proportional to the amount of fat mass⁹⁰. In stable depleted emphysematous patients with COPD, plasma levels of leptin were inversely related to dietary intake as well as to weight gain after nutritional therapy⁹¹. In addition, circulating leptin was found to be positively related to plasma soluble TNF-receptor 55 in stable patients with emphysema⁹¹. In addition to disturbances in energy balance, skeletal muscle dysfunction characterized by the progressive loss of skeletal muscle mass as well as fiber-type redistribution contributes to weight loss in COPD⁹². In addition to airflow limitation and loss of alveolar structure, skeletal muscle wasting was demonstrated to play an important role in dyspnea and exercise limitation in COPD⁹³, and thus the decline of health status.

1.7 Treatment

Despite the burden of COPD, treatment of COPD is primarily symptomatic, essentially bronchodilators, and often unsatisfactory with no drugs currently available that halt the progressive course of airflow limitation in COPD patients⁴. The regular use of glucocorticosteroids is disappointing, as important elements of the inflammatory response were demonstrated to be insensitive after short-term treatment with inhaled steroids^{94,95}. In addition, long-term therapy resulted in an improvement in FEV₁ in the first months of treatment, but the rate of decline in FEV₁ was not retarded after that period⁹⁶⁻⁹⁹. To date, identification, reduction, and control of risk factors (tobacco smoke, occupational exposures, air pollution, and irritants) is the single and most (cost) effective way toward prevention and treatment of COPD⁴. Since cigarette smoking is the major risk for COPD worldwide, smoking prevention programs are being implemented and smoking cessation programs will be readily available and encouraged for individuals who smoke. Quitting smoking can prevent or delay the development of airflow limitation or reduce the age-related decline in FEV₁^{13,100,101}. However, a longitudinal follow-up study of smoking-induced lung density changes by high-resolution computed tomography demonstrated that the worsening airspace abnormality in the upper lung zones of smokers did not slow down with cessation of smoking¹⁰². In addition, other studies investigating airway inflammation reported that smoking cessation did not result in resolution of the inflammatory response in the airways^{45,103-106}. These observations suggest that there are perpetuating mechanisms that maintain the chronic inflammatory process once it has become established, and such mechanisms may account for the presentation of COPD in patients who have stopped smoking many years before their first symptoms develop. Thus, there is a great need to develop new and effective therapeutic interventions for COPD.

1.8 Summary

COPD is a complex heterogeneous respiratory disease that includes three different disease conditions (chronic bronchitis, small airway disease, and emphysema) and represents a major and growing health care problem. Risk factors for development of COPD include both host factors and environmental exposures, and cigarette smoking is widely recognized as the most important risk factor in industrialized countries. There is increasing evidence that COPD is associated with a chronic inflammatory response throughout the airways, lung parenchyma, and pulmonary vasculature, although the types of inflammatory cells may vary in the different compartments of the lung. Cellular inflammatory infiltrates and production of inflammatory mediators, proteinases, and reactive nitrogen and oxygen species are all thought to contribute to the pathologic changes characteristic for COPD. Furthermore, there is increasing evidence of systemic (i.e. extrapulmonary) effects in patients with COPD, namely systemic inflammation, oxidative stress, and skeletal muscle dysfunction, which have important negative influence on the quality of life. Despite the burden of COPD, there are only limited therapeutic strategies available for treatment of COPD, and development of novel therapies is hampered by absence of suitable animal models of COPD for pre-clinical drug testing.

2. COPD MODELING

Until a few years ago, there has been little interest in investigating the molecular and cell biology of COPD. The largely physiological definition of COPD has certainly contributed to this lack of knowledge. We have yet to understand many crucial issues involved in COPD, such as mechanisms of airflow obstruction, inflammatory cell recruitment, persistence of chronic inflammation, and abnormal repair, before new therapeutic targets can be identified. In addition, it is still unclear why only 10-20% of cigarette smokers will eventually develop clinically significant COPD.

As there are major similarities in lung development and lung structure between humans and other mammals, animal models are critical experimental tools to study the pathophysiology of COPD. The ultimate goal is then to translate information gained from the development of animal models to prevention as well as treatment strategies. In the past decades, different models in rodents (rat, mouse, hamster, guinea pig, rabbit) and sheep have been developed to study COPD, each with its specific characteristics, as discussed below. Furthermore, a COPD-like disorder is known to exist in horses¹⁰⁷. It is, however, appropriate to note that the definition of emphysema in experimental animal models is only pathological without physiological descriptions¹⁰⁸. In the last decade, especially mice are widely used in experimental models. Advantages of the mouse over other experimental animals include the capacity to manipulate germline transmission of genetic information, a rapid reproductive cycle, and large litter sizes. In

addition, there is large knowledge of mouse biology, antibody and cDNA probes for the mouse are abundant, and mice are relatively cheap compared to other experimental animals¹⁰⁹. However, to date there are no satisfactory animal models mimicking both chronic inflammation and pathologic changes in COPD that are suitable for early drug testing.

2.1 Elastase-induced emphysema

Thirty-five years ago Gross demonstrated that instillation of papain into experimental animals resulted in profound emphysema¹¹⁰. Since Gross' initial experiments, investigators have instilled a variety of elastolytic enzymes, including pancreatic elastase¹¹¹, neutrophil elastase^{112,113}, and proteinase-3¹¹⁴, into the lungs of many small and large animals, resulting in panacinar emphysema. In contrast, instillation of non-elastolytic enzymes, such as bacterial collagenase, did not cause emphysema¹¹⁵. Disadvantages of these models, however, are that alveolar destruction by elastolytic enzymes occurs generally in absence of cellular inflammatory infiltrates in the lung. In addition, identification of proteinases and events upstream of proteinase release involved in cigarette smoke-induced emphysema is impossible. Nevertheless, the elastase model remained in use because of its relative simplicity and the fact that it allowed for first-order approximation for study of downstream events, particularly alveolar repair. For example, elastase instillation has been used to demonstrate that retinoic acid has the capacity to promote alveolarization and lung repair in adult rats¹¹⁶.

2.2 Cigarette smoke-related COPD

A variety of experimental animals have been exposed to cigarette smoke over the years using various protocols. Pathologic changes similar to those observed in COPD patients have been found in cigarette smoke-exposed mice, including low-grade chronic inflammation and destruction of alveolar structures¹¹⁷. On the other hand, mice do not cough up sputum after smoking, probably due to subtle differences in airway structure between man and mice¹¹⁵. If these pathologic changes are associated with abnormal pulmonary function or gas-exchange abnormalities, is still unknown and awaits further study. A disadvantage of cigarette-smoke exposure studies is the time needed to produce lesions, up to 12 month in some species. In addition, cigarette smoke is a complex mixture of over 4000 different chemical compounds, including free radicals and oxidants, which makes it difficult to unravel exact mechanisms of cigarette smoke-induced inflammatory processes. However, the cigarette-smoke model is popular, because smoking is the major risk factor to develop COPD. In addition, cigarette smoke-related changes have been demonstrated to be strain-dependent in mice¹¹⁸. This provides a unique opportunity to uncover genes involved in susceptibility to COPD.

2.3 Chemical-induced emphysema

A variety of chemicals and irritants have been used in experimental animals to generate COPD. Cadmium chloride is extensively used to generate

emphysematous lesions, which was found to be secondary to fibrotic processes¹¹⁹. In addition, experimental models exploring oxidants like nitrogen dioxide and ozone show that these components cause airway changes, such as epithelial cell injury and cilia loss, and mild focal emphysema¹²⁰. Furthermore, exposure studies to inorganic dusts like silica and coal dust demonstrated focal emphysema without changes in airway structures, associated with complex inflammation (neutrophilia) and oxidant injury with connective tissue breakdown¹²¹. Finally, lipopolysaccharide (LPS) of Gram-negative bacteria is used in experimental animals to generate COPD-like pathology. This will be discussed below (§3.2).

2.4 Genetic models of airspace enlargement

Natural mutations

Several spontaneous mutant mouse strains develop airspace enlargement. These are usually developmental abnormalities rather than destruction of mature lung tissue characterizing emphysema. Tight skin (Tsk+/-) mice have a mutation in fibrillin-1, which is involved in elastic fiber assembly¹²². These mice have abnormal air-space development and progressive alveolar enlargement with age. Pallid mice (pa/pa) develop mild emphysema late in life¹²³, but the cause for this is unknown. Blotchy mice have enlarged air spaces believed to be a result of abnormal connective tissue or cross-linking¹²⁴. More recently, this mutation has been localized to abnormal RNA processing of the Menke gene on the X chromosome mottled locus¹²⁴, but the relationship with emphysema remains unknown. Beige mice (bg) have a defect in formation of primary granules^{125,126}. It remains controversial whether they produce normal levels of serine proteinases and have the capacity to develop emphysema.

Transgenic mice

Just over one decade ago, techniques to achieve germline transmission of genetic material in mice (transgenic technology) drastically changed the approach to biological questions, and can be used to address questions of function of individual mediators. Introduction of a linear DNA fragment (transgene) into the pronucleus of embryos or embryonic stem cells allows study of either the pattern of expression of that gene, or the biological consequences of overexpression of the protein encoded by the gene in specific tissues (gain of function/overexpression model)¹⁰⁹. More recently, targeted mutagenesis by homologous recombination in embryonic stem cells has allowed investigators to generate strains of mice that lack individual proteins, providing specific loss of function models (loss of function/knock-out model)¹⁰⁹.

Several transgenic mice overexpressing mediators involved in inflammatory processes were shown to develop emphysematous changes. For example, mice overexpressing collagenase-1 (MMP-1) show enlarged air spaces¹²⁷, but it is unclear whether this is because of the destruction of collagen or interference with lung growth and development. In addition, lungs of transgenic mice overexpressing specific proinflammatory cytokines – inducible or not – in alveolar

type II cells (TNF- α ¹²⁸) or airway epithelial cells (IFN- γ , IL-6, IL-11, or IL-13¹²⁹⁻¹³¹) display enormous alveolar destruction, accompanied by profound ongoing inflammation in airways and lung parenchyma, the pattern of which is cytokine-dependent. In case of IL-13, the increase in lung size, alveolar size, lung compliance, and pulmonary inflammation was shown to be dependent on the chemokine receptor CCR2, whereas mucus cell metaplasia was not¹³².

Knock-out strains generated by targeted mutagenesis pointed at the important role of growth factors in alveolar septation. For example, lungs of FGFR-3/FGFR-4 double knock-out mice (deficient in both fibroblast growth factor receptors 3 and 4) are normal at birth but then fail to undergo alveogenesis¹³³. In addition, gradual appearance of emphysema with age has been described in surfactant protein D (SP-D) knock-out mice¹³⁴ and in mice lacking TIMP-3¹³⁵. In both cases, MMPs were implicated as mediators of tissue destruction. Furthermore, the *klotho* mouse (generated due to a failed transgenic experiment to develop a model of hypertension) displays features of premature aging, including a short lifespan, infertility, arteriosclerosis, skin atrophy, osteoporosis, and "emphysema"¹³⁶. It is, however, not yet clear whether *klotho* mice have abnormal development or truly premature aging resulting in emphysematous changes.

The combination of gene targeting with exposure models provides a unique opportunity to perform highly controlled experiments that differ with respect to expression of a single protein in mammals. In that way, strains of mice deficient in individual candidate genes can be compared to determine their contribution to the development of emphysema in response to cigarette smoke. For example, exposure of macrophage elastase (MMP-12) knock-out mice and wild-type littermates to chronic cigarette smoke exposure demonstrated that in contrast with wild-type mice, MMP-12 knock-out mice did not develop emphysema¹¹⁷. In addition, MMP-12 knock-out mice also failed to recruit macrophages into their lungs in response to cigarette smoke, hereby suggesting that the macrophage and its matrix metalloproteinases may be important in emphysematous changes, at least in mice.

2.5 Summary

Emphysema can be modelled in many ways. Exogenous administration of proteinases, chemicals, particulates, and exposure to cigarette smoke was demonstrated to result in features characteristic of human emphysema. In addition, airspace enlargement during development and throughout life was demonstrated in genetic manipulated mice (both gain and loss of function models). The use of animal models is crucial to address the many unsolved issues in COPD, such as mechanisms of airflow obstruction, inflammatory cell recruitment, persistence of chronic inflammation, and abnormal repair. To date, however, no satisfactory animal models mimicking both chronic inflammation and pathologic changes in COPD are available that are suitable for early drug testing.

3. LIPOPOLYSACCHARIDE (LPS) AND COPD

As outlined in §1.2, the major environmental factor that predisposes patients to COPD is chronic exposure to cigarette smoke. Recently, high levels of bioactive lipopolysaccharide (LPS) are reported to be present in cigarette smoke¹³⁷. LPS is a major proinflammatory constituent present in the outer cell wall of all Gram-negative bacteria. In addition, previous studies have revealed that LPS is ubiquitously present in high concentrations as contaminant on natural airborne particles, including organic dusts, air pollution, and house dusts¹³⁸⁻¹⁴².

Figure 2. The structure of LPS is composed of a hydrophobic lipophilic region (lipid A) and a covalently linked hydrophilic polysaccharide part consisting of an O-specific chain and a core region¹⁴³.

3.1 Structure and signal-transduction pathway of LPS

LPS is an extremely heat-stable amphiphilic molecule composed of a hydrophobic lipophilic region (lipid A) and a covalently linked hydrophilic poly- or oligosaccharide portion (Figure 2)¹⁴³. The lipid A part, which is highly conserved among LPS of different bacteria, is responsible for most of the biological effects of LPS due to the specific and often highly sensitive recognition of lipid A by numerous cellular and humoral components of innate immunity. Since LPS is present in all Gram-negative bacteria, the host recognition of and response to this glycolipid provides protection against a wide range of microorganisms¹⁴³.

The primary target cells of LPS in mammalian species are the professional phagocytes of the innate immunity system (ie peripheral macrophages, tissue macrophages, and neutrophils). Extensive studies on the molecular mechanisms of LPS recognition and signaling have elucidated that phagocyte activation by extracellularly supplied LPS or lipid A is initialized by the combined actions of LPS binding protein (LBP), the membrane forms of CD14 (mCD14), and the recently identified Toll-like receptor (TLR)-4*MD2 complex on the cellular surface (Figure 3). As a result, rapid and coordinated activation of various intracellular signaling pathways including activation of the major MAP kinase cascades and translocation of NF- κ B will lead to the secretion of a wide spectrum of endogenous mediators, such as pro-inflammatory cytokines, chemokines, lipid-derived mediators, and reactive oxygen species¹⁴³. By means of the soluble form of CD14 antigen (sCD14), LPS may also activate constitutively CD14 negative cells such as

dendritic cells, vascular endothelial and smooth muscle cells as well as epithelial cells and fibroblasts, most of which, in case of human origin, have been demonstrated to express TLR-4. Recently, Nod1 and Nod2 were discovered as independent cytosolic counterparts of TLRs, acting in activation of NF- κ B via binding of intracellular LPS¹⁴⁴. The endogenous mediators released during this early phase of LPS-induced activation of innate immunity initiate a complex network of secondary reactions. These include the activation of the complement system (both classical and lectin pathways), the stimulation of acute-phase protein secretion by hepatocytes, the activation of lymphocytes, platelets, basophils, mast cells, and eosinophils, an elevated hematopoietic activity of the bone marrow, an increased systemic procoagulant state, and activation of the neuro-endocrine system¹⁴⁵.

Figure 3. LBP/CD14/TLR4/MD2 signal transduction pathway of LPS results in translocation of NF- κ B to the nucleus and subsequent gene expression of cytokines, chemokines, and adhesion molecules.

3.2 LPS exposure studies

Human studies

In ambient breathing air, LPS is measured at very low concentrations (± 0.4 ng/m³)¹⁴⁶. Under these conditions, the lung has efficient defense mechanisms against airborne LPS, including 1) the physical removal by the mucociliary system and cough; 2) the presence of broad-spectrum anti-microbial agents in the epithelial lining fluid; 3) the activation of resident alveolar macrophages; and 4) the epithelial barrier itself²³.

Exposure to significant levels of LPS, however, is associated with the development and/or progression of chronic lung diseases. Epidemiological studies have shown that the concentration of inhaled LPS in the bioaerosol was strongly associated with airflow obstruction in cotton workers¹⁴⁷, agricultural workers¹⁴⁸, and fiberglass workers¹⁴⁹. In fact, the concentration of LPS in the bioaerosol was found to be the most important occupational exposure associated with the

development¹⁹ and progression¹⁴⁸ of chronic airway disease in agricultural workers. Moreover, healthy study subjects challenged with dust from animal confinement buildings developed airflow obstruction and an increase in the serum concentration of neutrophils and IL-6, all of which were strongly associated with the concentration of endotoxin (not dust) in the bioaerosol¹⁵⁰.

Experimentally, inhalation of LPS induces dose-related clinical symptoms, such as fever and fatigue, reversible airflow obstruction and activation of the innate immune system in previously unexposed healthy study subjects^{151,152}. Within 4-6 hours following inhalation of LPS or organic dusts containing LPS, an influx of neutrophils and lymphocytes was found in BAL fluid with increased levels of inflammatory mediators (TNF- α , IL-1 β , IL-6, IL-8, and fibronectin)¹⁵³⁻¹⁵⁵. Circulating levels of TNF- α and CRP were also raised^{151,156}. As *in vitro* studies have demonstrated that respiratory epithelial cells secrete a variety of inflammatory mediators in response to LPS^{44,157}, the respiratory epithelium may be an important source of these mediators in BAL fluid after LPS inhalation in healthy subjects.

There is a considerable inter-individual variability in the sensitivity to LPS by inhalation. Asthmatics¹⁵⁸ and individuals with chronic bronchitis¹⁵⁹ respond more sensitive to LPS as they develop airflow obstruction at lower concentrations of inhaled LPS compared to healthy subjects. In addition, the concentration of airborne endotoxin in the domestic setting was reported to be associated with the clinical severity of asthma in a dose-dependent manner¹⁴². Recently, co-segregating missense mutations (Asp299Gly and Thr399Ile) affecting the extracellular domain of the TLR4 receptor were found to be associated with a blunted response to inhaled LPS in humans. This study demonstrates that gene-sequence changes can alter the ability of the host to respond to environmental stress¹⁶⁰.

Animal models

In view of the potent inflammatory effects of LPS, the role of LPS in lung diseases was further studied in experimental animal models. Resident alveolar macrophages are considered to be the most important effector cells to pulmonary LPS exposure. Activation of alveolar macrophages by a single LPS dose results in an inflammatory cascade defined by regulated expression of a variety of genes in the lung, including early response cytokines (TNF- α , IL-1 α , IL-1 β , IL-6), chemokines (MIP-1 α , MIP-1 β , MIP-2, CINC), and endothelial leukocyte adhesion molecules (ICAM-1, selectins)¹⁶¹⁻¹⁶⁹. The transcription of many of these genes is mediated by the NF- κ B family of transcription factors, and nuclear translocation of the NF- κ B RelA subunit was shown to be critical to inducing the gene expression mediating neutrophil emigration elicited by LPS in the lungs¹⁷⁰. The increased gene expression consequently results into profound, but transient influx of neutrophils into the interstitium and air spaces¹⁷¹. This neutrophilia plays a prominent role in the host defence against pathogens, but is also considered to be responsible for the pulmonary injury, manifested by increased lung vascular permeability, edema and cell death, as neutrophils release histotoxic substances, including proteases and reactive oxygen species^{172,173}. In this respect, programmed cell death

(apoptosis) plays a crucial role in limiting the inflammatory response and clearance of neutrophils from inflamed tissue^{174,175}.

In addition to alveolar macrophages, airway and alveolar epithelial cells contribute to the innate immune response in response to pulmonary LPS exposure. LPS exposure results in increased production of direct antimicrobial molecules, mucins¹⁷⁶, and NO by airway epithelium, and surfactant proteins and acute-phase reactants by alveolar epithelium¹⁷⁷⁻¹⁸⁰. In concert with the upregulation of this antimicrobial defense, LPS exposure initiates an inflammatory response in epithelial cells. *In vitro* studies demonstrated that primary respiratory epithelial cell cultures as well as cell lines stimulated with LPS produce a variety of chemoattractants, cytokines, and growth factors (reviewed by Fehrenbach¹⁸¹).

Studies investigating chronic LPS exposure are limited, and primarily focus on pathologic changes. In a series of studies, Stolk and colleagues reported that repeated intratracheal LPS instillation in hamsters resulted in bronchial mucus cell hyperplasia and profound parenchymal destruction. Inhibition of endogenous neutrophil elastase, released by recruited neutrophils as a consequence of the LPS instillations, was shown to reduce the extent of LPS-induced emphysema¹⁸²⁻¹⁸⁶. Consistent with the hamster studies, destruction of alveolar structures by repeated pulmonary LPS exposure was shown in guinea pigs¹⁸⁷⁻¹⁸⁹, rats¹⁹⁰, and mice^{191,192}. In addition, airway wall thickening was noted after long-term exposure to LPS-contaminated corn dust extract in LPS-sensitive mice (C3H/HeBFeJ), but not in LPS-resistant mice (C3H/HeJ)¹⁹³. In contrast to the pathologic changes induced by repeated LPS exposure to the lungs, the chronic inflammatory response is not well defined. A recent study reported peribronchial cellular infiltrates as well as neutrophilic infiltration in airways, parenchyma and vasculature within 1 day after chronic LPS exposure in mice¹⁹¹. In view of this LPS-induced neutrophilia, depletion of neutrophils was shown to diminish LPS-induced airway wall thickening¹⁹².

3.3 Summary

The respiratory system is continuously exposed to bacterial LPS due to inhalation of airborne particles, including cigarette smoke and organic dusts. Experimentally, inhalation of LPS induces dose-related clinical symptoms, such as fever and fatigue, reversible airflow obstruction and activation of the innate immune system. There is a considerable inter-individual variability in the sensitivity to LPS by inhalation, with asthmatics and individuals with chronic bronchitis responding more sensitive than healthy subjects. Furthermore, chronic exposure to significant levels of LPS is known to be associated with the development and/or progression of chronic inflammatory lung diseases.

In this view, the role of LPS in lung diseases was further studied in experimental animal models. Extensive studies have demonstrated that single exposure of LPS to the lung activates alveolar macrophages via LPS binding protein (LBP)/CD14/Toll-like receptor (TLR)-4/MD2 dependent pathway to produce specific cytokines, associated with transient neutrophil influx and vascular permeability. Studies investigating chronic LPS exposure are limited, and primarily

focus on pathologic changes being bronchial mucus cells hyperplasia and parenchymal destruction.

REFERENCES

1. ATS. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1995;152:S77-121.
2. Siafakas NM, Vermeire P, Pride NB, Paoletti P, Gibson J, Howard P, Yernault JC, Decramer M, Higenbottam T, Postma DS, and et al. Optimal assessment and management of chronic obstructive pulmonary disease (COPD). The European Respiratory Society Task Force. *Eur Respir J* 1995;8:1398-1420.
3. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001;164:S28-38.
4. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, and Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001;163:1256-1276.
5. Barnes PJ. Chronic obstructive pulmonary disease. *N Engl J Med* 2000;343:269-280.
6. Van den Boom G, Van Schayck CP, Van Mollen MP, Tirimanna PR, Den Otter JJ, Van Grunsven PM, Buitendijk MJ, Van Herwaarden CL, and Van Weel C. Active detection of chronic obstructive pulmonary disease and asthma in the general population. Results and economic consequences of the DIMCA program. *Am J Respir Crit Care Med* 1998;158:1730-1738.
7. Rutten-van Molken MP, Postma MJ, Joore MA, Van Genugten ML, Leidl R, and Jager JC. Current and future medical costs of asthma and chronic obstructive pulmonary disease in The Netherlands. *Respir Med* 1999;93:779-787.
8. Mahadeva R, and Lomas DA. Genetics and respiratory disease. 2. Alpha 1-antitrypsin deficiency, cirrhosis and emphysema. *Thorax* 1998;53:501-505.
9. Lieberman J, Winter B, and Sastre A. Alpha 1-antitrypsin Pi-types in 965 COPD patients. *Chest* 1986;89:370-373.
10. Fletcher C, and Peto R. The natural history of chronic airflow obstruction. *Br Med J* 1977;1:1645-1648.
11. Barnes PJ. Chronic obstructive pulmonary disease: new opportunities for drug development. *Trends Pharmacol Sci* 1998;19:415-423.
12. Sherrill DL, Holberg CJ, Enright PL, Lebowitz MD, and Burrows B. Longitudinal analysis of the effects of smoking onset and cessation on pulmonary function. *Am J Respir Crit Care Med* 1994;149:591-597.
13. Scanlon PD, Connett JE, Waller LA, Altose MD, Bailey WC, and Buist AS. Smoking cessation and lung function in mild-to-moderate chronic obstructive pulmonary disease. The Lung Health Study. *Am J Respir Crit Care Med* 2000;161:381-390.
14. Anthonisen NR, Connett JE, and Murray RP. Smoking and lung function of Lung Health Study participants after 11 years. *Am J Respir Crit Care Med* 2002;166:675-679.
15. Anthonisen NR, Connett JE, Enright PL, and Manfreda J. Hospitalizations and mortality in the Lung Health Study. *Am J Respir Crit Care Med* 2002;166:333-339.
16. Kauffmann F, Drouet D, Lellouch J, and Brille D. Twelve years spirometric changes among Paris area workers. *Int J Epidemiol* 1979;8:201-212.
17. Vogelzang PF, van der Gulden JW, Folgering H, Kolk JJ, Heederik D, Preller L, Tielen MJ, and van Schayck CP. Endotoxin exposure as a major determinant of lung function decline in pig farmers. *Am J Respir Crit Care Med* 1998;157:15-18.
18. Smid T, Heederik D, Houba R, and Quanjer PH. Dust- and endotoxin-related acute lung function changes and work-related symptoms in workers in the animal feed industry. *Am J Ind Med* 1994;25:877-888.
19. Schwartz DA, Thorne PS, Yagla SJ, Burmeister LF, Olenchock SA, Watt JL, and Quinn TJ. The role of endotoxin in grain dust-induced lung disease. *Am J Respir Crit Care Med* 1995;152:603-608.

20. Oxman AD, Muir DC, Shannon HS, Stock SR, Hnizdo E, and Lange HJ. Occupational dust exposure and chronic obstructive pulmonary disease. A systematic overview of the evidence. *Am Rev Respir Dis* 1993;148:38-48.
21. Sunyer J. Urban air pollution and chronic obstructive pulmonary disease: a review. *Eur Respir J* 2001;17:1024-1033.
22. Buist AS, and Vollmer WM. 1994. Smoking and other risk factors. In J.F. Murray, and J.A. Nadel, editors. *Textbook of respiratory medicine*, ed. Saunders, W.B., Philadelphia. 1259-1287.
23. Whitsett JA. Intrinsic and innate defenses in the lung: intersection of pathways regulating lung morphogenesis, host defense, and repair. *J Clin Invest* 2002;109:565-569.
24. Finkelstein R, Fraser RS, Ghezzi H, and Cosio MG. Alveolar inflammation and its relation to emphysema in smokers. *Am J Respir Crit Care Med* 1995;152:1666-1672.
25. Saetta M, Di Stefano A, Turato G, Facchini FM, Corbino L, Mapp CE, Maestrelli P, Ciaccia A, and Fabbri LM. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;157:822-826.
26. Peleman RA, Ryttila PH, Kips JC, Joos GF, and Pauwels RA. The cellular composition of induced sputum in chronic obstructive pulmonary disease. *Eur Respir J* 1999;13:839-843.
27. Pesci A, Balbi B, Majori M, Cacciani G, Bertacco S, Alciato P, and Donner CF. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J* 1998;12:380-386.
28. Keatings VM, Collins PD, Scott DM, and Barnes PJ. Differences in interleukin-8 and tumor necrosis factor- α in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530-534.
29. O'Shaughnessy TC, Ansari TW, Barnes NC, and Jeffery PK. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am J Respir Crit Care Med* 1997;155:852-857.
30. Alexis NE, Hu SC, Zeman K, Alter T, and Bennett WD. Induced Sputum Derives from the Central Airways. Confirmation using a radiolabeled aerosol bolus delivery technique. *Am J Respir Crit Care Med* 2001;164:1964-1970.
31. Di Stefano A, Capelli A, Lusuadi M, Balbo P, Vecchio C, Maestrelli P, Mapp CE, Fabbri LM, Donner CF, and Saetta M. Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med* 1998;158:1277-1285.
32. Keatings VM, and Barnes PJ. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am J Respir Crit Care Med* 1997;155:449-453.
33. Betsuyaku T, Nishimura M, Takeyabu K, Tanino M, Venge P, Xu S, and Kawakami Y. Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Respir Crit Care Med* 1999;159:1985-1991.
34. Stockley RA. Neutrophils and the pathogenesis of COPD. *Chest* 2002;121:151S-155S.
35. Pettersen CA, and Adler KB. Airways inflammation and COPD: epithelial-neutrophil interactions. *Chest* 2002;121:142S-150S.
36. Woessner JF. 1998. *The Matrix Metalloproteinase Family*. Academic Press, San Diego.
37. Peinado VI, Barbera JA, Abate P, Ramirez J, Roca J, Santos S, and Rodriguez-Roisin R. Inflammatory reaction in pulmonary muscular arteries of patients with mild chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;159:1605-1611.
38. Lacoste JY, Bousquet J, Chanaz P, Van Vyve T, Simony-Lafontaine J, Lequeu N, Vic P, Enander I, Godard P, and Michel FB. Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 1993;92:537-548.
39. Pesci A, Majori M, Cuomo A, Borciani N, Bertacco S, Cacciani G, and Gabrielli M. Neutrophils infiltrating bronchial epithelium in chronic obstructive pulmonary disease. *Respir Med* 1998;92:863-870.
40. Saetta M, Baraldo S, Corbino L, Turato G, Braccioni F, Rea F, Cavallero G, Tropeano G, Mapp CE, Maestrelli P, Ciaccia A, and Fabbri LM. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;160:711-717.
41. Peinado VI, Barbera JA, Abate P, Ramirez J, Roca J, Santos S, and Rodriguez Roisin R. Inflammatory reaction in pulmonary muscular arteries of patients with mild chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;159:1605-1611.

42. Turato G, Zuin R, Miniati M, Baraldo S, Rea F, Beghe B, Monti S, Formichi B, Boschetto P, Harari S, Papi A, Maestrelli P, Fabbri LM, and Saetta M. Airway inflammation in severe chronic obstructive pulmonary disease: relationship with lung function and radiologic emphysema. *Am J Respir Crit Care Med* 2002;166:105-110.
43. Takizawa H. Airway epithelial cells as regulators of airway inflammation. *Int J Mol Med* 1998;1:367-378.
44. Diamond C, Legarda D, and Ryan LK. The innate immune response of the respiratory epithelium. *Immunol Rev* 2000;173:27-38.
45. Turato G, Di Stefano A, Maestrelli P, Mapp CE, Ruggieri MP, Roggeri A, Fabbri LM, and Saetta M. Effect of smoking cessation on airway inflammation in chronic bronchitis. *Am J Respir Crit Care Med* 1995;152:1262-1267.
46. Di Stefano A, Maestrelli P, Roggeri A, Turato G, Calabro S, Potena A, Mapp CE, Ciaccia A, Covacev L, Fabbri LM, and et al. Upregulation of adhesion molecules in the bronchial mucosa of subjects with chronic obstructive bronchitis. *Am J Respir Crit Care Med* 1994;149:803-810.
47. Traves SL, Culpitt SV, Russell RE, Barnes PJ, and Donnelly LE. Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax* 2002;57:590-595.
48. Capelli A, Di Stefano A, Gnemm I, Balbo P, Cerutti CG, Balbi B, Lusuardi M, and Donner CF. Increased MCP-1 and MIP-1beta in bronchoalveolar lavage fluid of chronic bronchitics. *Eur Respir J* 1999;14:160-165.
49. Hill AT, Bayley D, and Stockley RA. The interrelationship of sputum inflammatory markers in patients with chronic bronchitis. *Am J Respir Crit Care Med* 1999;160:893-898.
50. De Boer WJ, Sont JK, Van Schadewijk A, Stolk J, Van Krieken JH, and Hiemstra PS. Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *J Pathol* 2000;190:619-626.
51. De Boer WJ, van Schadewijk A, Sont JK, Sharma HS, Stolk J, Hiemstra PS, and Van Krieken JH. Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;158:1951-1957.
52. Vignola AM, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, la Rocca AM, Bellia V, Bonsignore G, and Bousquet J. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1997;156:591-599.
53. Saetta M, Mariani M, Panina-Bordignon P, Turato G, Buonsanti C, Baraldo S, Bellettato CM, Papi A, Corbetta L, Zuin R, Sinigaglia F, and Fabbri LM. Increased Expression of the Chemokine Receptor CXCR3 and Its Ligand CXCL10 in Peripheral Airways of Smokers with Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 2002;165:1404-1409.
54. Hogg JC, and Senior RM. Chronic obstructive pulmonary disease - part 2: pathology and biochemistry of emphysema. *Thorax* 2002;57:830-834.
55. Gottlieb DJ, Luisetti M, Stone PJ, Allegra L, Cantey-Kiser JM, Grassi C, and Snider GL. Short-term supplementation therapy does not affect elastin degradation in severe alpha(1)-antitrypsin deficiency. The American-Italian AATD Study Group. *Am J Respir Crit Care Med* 2000;162:2069-2072.
56. Ludwig PW, Schwartz BA, Hoidal JR, and Niewoehner DE. Cigarette smoking causes accumulation of polymorphonuclear leukocytes in alveolar septum. *Am Rev Respir Dis* 1985;131:828-830.
57. Handbook for proteolytic enzymes. 1998. In A.J. Barret, N.D. Rawlings, and F. Woessner, editors. ed. Academic Press, London, UK.
58. Turk V, Turk B, and Turk D. Lysosomal cysteine proteases: facts and opportunities. *Embo J* 2001;20:4629-4633.
59. Takeyabu K, Betsuyaku T, Nishimura M, Yoshioka A, Tanino M, Miyamoto K, and Kawakami Y. Cysteine proteinases and cystatin C in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Eur Respir J* 1998;12:1033-1039.
60. Piccioni PD, Kramps JA, Rudolphus A, Bulgheroni A, and Luisetti M. Proteinase/proteinase inhibitor imbalance in sputum sol phases from patients with chronic obstructive pulmonary disease. Suggestions for a key role played by antileukoprotease. *Chest* 1992;102:1470-1476.
61. Parks WC, and Shapiro SD. Matrix metalloproteinases in lung biology. *Respir Res* 2001;2:10-19.
62. Vignola AM, Riccobono L, Mirabella A, Profita M, Chanez P, Bellia V, Mautino G, D'Accardi P, Bousquet J, and Bonsignore G. Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-

- 1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1998;158:1945-1950.
63. Segura-Valdez L, Pardo A, Gaxiola M, Uhal BD, Becerril C, and Selman M. Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. *Chest* 2000;117:684-694.
 64. Cataldo D, Munaut C, Noel A, Frankenne F, Bartsch P, Foidart JM, and Louis R. MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. *Int Arch Allergy Immunol* 2000;123:259-267.
 65. Ohnishi K, Takagi M, Kurokawa Y, Satomi S, and Kontinen YT. Matrix metalloproteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema. *Lab Invest* 1998;78:1077-1087.
 66. Imai K, Dalal SS, Chen ES, Downey R, Schulman LL, Ginsburg M, and D'Armiento J. Human collagenase (matrix metalloproteinase-1) expression in the lungs of patients with emphysema. *Am J Respir Crit Care Med* 2001;163:786-791.
 67. Dekhuijzen PN, Aben KK, Dekker I, Aarts LP, Wielders PL, van Herwaarden CL, and Bast A. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1996;154:813-816.
 68. Maziak W, Loukides S, Culpitt S, Sullivan P, Kharitonov SA, and Barnes PJ. Exhaled nitric oxide in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;157:998-1002.
 69. Report NWW. 2001.Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD).
 70. Niewoehner DE, Kleinerman J, and Rice DB. Pathologic changes in the peripheral airways of young cigarette smokers. *N Engl J Med* 1974;291:755-758.
 71. Matsuba K, and Thurlbeck WM. The number and dimensions of small airways in emphysematous lungs. *Am J Pathol* 1972;67:265-275.
 72. Hogg JC, Macklem PT, and Thurlbeck WM. Site and nature of airway obstruction in chronic obstructive lung disease. *N Engl J Med* 1968;278:1355-1360.
 73. Wright JL, Lawson L, Pare PD, Hooper RO, Peretz DI, Nelems JM, Schulzer M, and Hogg JC. The structure and function of the pulmonary vasculature in mild chronic obstructive pulmonary disease. The effect of oxygen and exercise. *Am Rev Respir Dis* 1983;128:702-707.
 74. Sekhon HS, Wright JL, and Churg A. Cigarette smoke causes rapid cell proliferation in small airways and associated pulmonary arteries. *Am J Physiol* 1994;267:L557-563.
 75. Peinado VI, Barbera JA, Ramirez J, Gomez FP, Roca J, Jover L, Gimferrer JM, and Rodriguez-Roisin R. Endothelial dysfunction in pulmonary arteries of patients with mild COPD. *Am J Physiol* 1998;274:L908-913.
 76. Kasahara Y, Tudor RM, Cool CD, Lynch DA, Flores SC, and Voelkel NF. Endothelial Cell Death and Decreased Expression of Vascular Endothelial Growth Factor and Vascular Endothelial Growth Factor Receptor 2 in Emphysema. *Am J Respir Crit Care Med* 2001;163:737-744.
 77. Schols AM, Buurman WA, Staal van den Brekel AJ, Dentener MA, and Wouters EF. Evidence for a relation between metabolic derangements and increased levels of inflammatory mediators in a subgroup of patients with chronic obstructive pulmonary disease. *Thorax* 1996;51:819-824.
 78. Eid AA, Ionescu AA, Nixon LS, Lewis-Jenkins V, Matthews SB, Griffiths TL, and Shale DJ. Inflammatory response and body composition in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164:1414-1418.
 79. De Godoy I, Donahoe M, Calhoun WJ, Mancino J, and Rogers RM. Elevated TNF-alpha production by peripheral blood monocytes of weight-losing COPD patients. *Am J Respir Crit Care Med* 1996;153:633-637.
 80. Di Francia M, Barbier D, Mege JL, and Orehek J. Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1994;150:1453-1455.
 81. Noguera A, Busquets X, Sauleda J, Villaverde JM, MacNee W, and Agusti AG. Expression of adhesion molecules and G proteins in circulating neutrophils in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;158:1664-1668.
 82. Rahman I, Morrison D, Donaldson K, and MacNee W. Systemic oxidative stress in asthma, COPD, and smokers. *Am J Respir Crit Care Med* 1996;154:1055-1060.

83. Sauleda J, Garcia-Palmer FJ, Gonzalez G, Palou A, and Agusti AG. The activity of cytochrome oxidase is increased in circulating lymphocytes of patients with chronic obstructive pulmonary disease, asthma, and chronic arthritis. *Am J Respir Crit Care Med* 2000;161:32-35.
84. Dentener MA, Creutzberg EC, Schols AM, Mantovani A, van't Veer C, Buurman WA, and Wouters EF. Systemic anti-inflammatory mediators in COPD: increase in soluble interleukin 1 receptor II during treatment of exacerbations. *Thorax* 2001;56:721-726.
85. Takabatake N, Nakamura H, Abe S, Hino T, Saito H, Yuki H, Kato S, and Tomoike H. Circulating leptin in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;159:1215-1219.
86. Takabatake N, Nakamura H, Abe S, Inoue S, Hino T, Saito H, Yuki H, Kato S, and Tomoike H. The relationship between chronic hypoxemia and activation of the tumor necrosis factor- α system in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;161:1179-1184.
87. Dahl M, Tybjaerg-Hansen A, Vestbo J, Lange P, and Nordestgaard BG. Elevated plasma fibrinogen associated with reduced pulmonary function and increased risk of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164:1008-1011.
88. Engelen MP, Schols AM, Lamers RJ, and Wouters EF. Different patterns of chronic tissue wasting among patients with chronic obstructive pulmonary disease. *Clin Nutr* 1999;18:275-280.
89. Campfield LA, Smith FJ, and Burn P. The OB protein (leptin) pathway—a link between adipose tissue mass and central neural networks. *Horm Metab Res* 1996;28:619-632.
90. Huang L, and Li C. Leptin: a multifunctional hormone. *Cell Res* 2000;10:81-92.
91. Schols AM, Creutzberg EC, Buurman WA, Campfield LA, Saris WH, and Wouters EF. Plasma leptin is related to proinflammatory status and dietary intake in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;160:1220-1226.
92. Gosker HR, van Mameren H, van Dijk PJ, Engelen MP, van der Vusse GJ, Wouters EF, and Schols AM. Skeletal muscle fibre-type shifting and metabolic profile in patients with chronic obstructive pulmonary disease. *Eur Respir J* 2002;19:617-625.
93. ATS. Skeletal muscle dysfunction in chronic obstructive pulmonary disease. A statement of the American Thoracic Society and European Respiratory Society. *Am J Respir Crit Care Med* 1999;159:S1-40.
94. Culpitt SV, Maziak W, Loukidis S, Nightingale JA, Matthews JL, and Barnes PJ. Effect of high dose inhaled steroid on cells, cytokines, and proteases in induced sputum in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;160:1635-1639.
95. Keatings VM, Jatakanon A, Worsdell YM, and Barnes PJ. Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *Am J Respir Crit Care Med* 1997;155:542-548.
96. Paggiaro PL, Dahle R, Bakran I, Frith L, Hollingworth K, and Efthimiou J. Multicentre randomised placebo-controlled trial of inhaled fluticasone propionate in patients with chronic obstructive pulmonary disease. International COPD Study Group [see comments]. *Lancet* 1998;351:773-780.
97. Burge PS, Calverley PM, Jones PW, Spencer S, Anderson JA, and Maslen TK. Randomised, double blind, placebo controlled study of fluticasone propionate in patients with moderate to severe chronic obstructive pulmonary disease: the ISOLDE trial. *Bmj* 2000;320:1297-1303.
98. Vestbo J, Sorensen T, Lange P, Brix A, Torre P, and Viskum K. Long-term effect of inhaled budesonide in mild and moderate chronic obstructive pulmonary disease: a randomised controlled trial. *Lancet* 1999;353:1819-1823.
99. Pauwels RA, Lofdahl CG, Laitinen LA, Schouten JP, Postma DS, Pride NB, and Ohlsson SV. Long-term treatment with inhaled budesonide in persons with mild chronic obstructive pulmonary disease who continue smoking. European Respiratory Society Study on Chronic Obstructive Pulmonary Disease. *N Engl J Med* 1999;340:1948-1953.
100. Anthonisen NR, Connett JE, Kiley JP, Altose MD, Bailey WC, Buist AS, Conway WA, Jr., Enright PL, Kanner RE, O'Hara P, and et al. Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV1. The Lung Health Study. *Jama* 1994;272:1497-1505.
101. Kanner RE, Connett JE, Williams DE, and Buist AS. Effects of randomized assignment to a smoking cessation intervention and changes in smoking habits on respiratory symptoms in

- smokers with early chronic obstructive pulmonary disease: the Lung Health Study. *Am J Med* 1999;106:410-416.
102. Soejima K, Yamaguchi K, Kohda E, Takeshita K, Ito Y, Mastubara H, Oguma T, Inoue T, Okubo Y, Amakawa K, Tatenno H, and Shiomi T. Longitudinal follow-up study of smoking-induced lung density changes by high-resolution computed tomography. *Am J Respir Crit Care Med* 2000;161:1264-1273.
 103. Mullen JB, Wright JL, Wiggs BR, Pare PD, and Hogg JC. Structure of central airways in current smokers and ex-smokers with and without mucus hypersecretion: relationship to lung function. *Thorax* 1987;42:843-848.
 104. Yamamoto C, Yoneda T, Yoshikawa M, Fu A, Tokuyama T, Tsukaguchi K, and Narita N. Airway inflammation in COPD assessed by sputum levels of interleukin-8. *Chest* 1997;112:505-510.
 105. Rutgers SR, Postma DS, ten Hacken NH, Kauffman HF, van Der Mark TW, Koeter GH, and Timens W. Ongoing airway inflammation in patients with COPD who do not currently smoke. *Thorax* 2000;55:12-18.
 106. Willemse BW, Ten Hacken NH, Rutgers B, G. Li, Postma DS, and Timens W. The effect of one year smoking cessation on neutrophilic inflammation in smokers with and without COPD. *Am J Respir Crit Care Med* 2002;165:A463.
 107. Robinson NE, Derksen FJ, Olszewski MA, and Buechner-Maxwell VA. The pathogenesis of chronic obstructive pulmonary disease of horses. *Br Vet J* 1995;152:283-306.
 108. Snider GL, J. K, Thurlbeck WM, and al. e. The definition of emphysema: report of a National Heart, Lung, and Blood Institute; Devision of Lung Diseases Workshop. *Am Rev Respir Dis* 1985;132:182-185.
 109. Shapiro SD. Mighty mice: transgenic technology "knocks out" questions of matrix metalloproteinase function. *Matrix Biol* 1997;15:527-533.
 110. Gross PE, Pfltzar E, Tolker M, Babyak M, and Kaschak M. Experimental emphysema: its production with papain in normal and silicotic rats. *Arch. Environ. Health* 1965;11:50-58.
 111. Kuhn C, Yu SY, Chraplyvy M, Linder HE, and Senior RM. The induction of emphysema with elastase. II. Changes in connective tissue. *Lab Invest* 1976;34:372-380.
 112. Senior RM, Tegner H, Kuhn C, Ohlsson K, Starcher BC, and Pierce JA. The induction of pulmonary emphysema with human leukocyte elastase. *Am Rev Respir Dis* 1977;116:469-475.
 113. Janoff A, Sloan B, Weinbaum G, Damiano V, Sandhaus RA, Elias J, and Kimbel P. Experimental emphysema induced with purified human neutrophil elastase: tissue localization of the instilled protease. *Am Rev Respir Dis* 1977;115:461-478.
 114. Kao RC, Wehner NG, Skubitz KM, Gray BH, and Hoidal JR. Proteinase 3. A distinct human polymorphonuclear leukocyte proteinase that produces emphysema in hamsters. *J Clin Invest* 1988;82:1963-1973.
 115. Shapiro SD. Animal models for chronic obstructive pulmonary disease: age of klotho and marlboro mice. *Am J Respir Cell Mol Biol* 2000;22:4-7.
 116. Massaro GD, and Massaro D. Retinoic acid treatment abrogates elastase-induced pulmonary emphysema in rats. *Nat Med* 1997;3:675-677.
 117. Hautamaki RD, Kobayashi DK, Senior RM, and Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 1997;277:2002-2004.
 118. Cavarra E, Bartalesi B, Lucattelli M, Fineschi S, Lunghi B, Gambelli F, Ortiz LA, Martorana PA, and Lungarella G. Effects of Cigarette Smoke in Mice with Different Levels of alpha(1)- Proteinase Inhibitor and Sensitivity to Oxidants. *Am J Respir Crit Care Med* 2001;164:886-890.
 119. Snider GL, Lucey EC, Faris B, Jung-Legg Y, Stone PJ, and Franzblau C. Cadmium-chloride-induced air-space enlargement with interstitial pulmonary fibrosis is not associated with destruction of lung elastin. Implications for the pathogenesis of human emphysema. *Am Rev Respir Dis* 1988;137:918-923.
 120. Chitano P, Hosselet JJ, Mapp CE, and Fabbri LM. Effect of oxidant air pollutants on the respiratory system: insights from experimental animal research. *Eur Respir J* 1995;8:1357-1371.
 121. Li K, Keeling B, and Churg A. Mineral dusts cause elastin and collagen breakdown in the rat lung: a potential mechanism of dust-induced emphysema. *Am J Respir Crit Care Med* 1996;153:644-649.
 122. Kiely CM, Raghunath M, Siracusa LD, Sherratt MJ, Peters R, Shuttleworth CA, and Jimenez SA. The Tight skin mouse: demonstration of mutant fibrillin-1 production and assembly into abnormal microfibrils. *J Cell Biol* 1998;140:1159-1166.

123. Keil M, Lungarella G, Cavarra E, van Even P, and Martorana PA. A scanning electron microscopic investigation of genetic emphysema in tight-skin, pallid, and beige mice, three different C57 BL/6J mutants. *Lab Invest* 1996;74:353-362.
124. Fisk DE, and Kuhn C. Emphysema-like changes in the lungs of the blotchy mouse. *Am Rev Respir Dis* 1976;113:787-797.
125. Perou CM, Justice MJ, Pryor RJ, and Kaplan J. Complementation of the beige mutation in cultured cells by episomally replicating murine yeast artificial chromosomes. *Proc Natl Acad Sci U S A* 1996;93:5905-5909.
126. Nagle DL, Karim MA, Woolf EA, Holmgren L, Bork P, Misumi DJ, McGrail SH, Dussault BJ, Jr., Perou CM, Boissy RE, Duyk GM, Spritz RA, and Moore KJ. Identification and mutation analysis of the complete gene for Chediak-Higashi syndrome. *Nat Genet* 1996;14:307-311.
127. D'Armiento J, Dafal SS, Okada Y, Berg RA, and Chada K. Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema. *Cell* 1992;71:955-961.
128. Fujita M, Shannon JM, Irvin CG, Fagan KA, Cool C, Augustin A, and Mason RJ. Overexpression of tumor necrosis factor-alpha produces an increase in lung volumes and pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L39-49.
129. Wang Z, Zheng T, Zhu Z, Homer RJ, Riese RJ, Chapman HA, Jr., Shapiro SD, and Elias JA. Interferon gamma induction of pulmonary emphysema in the adult murine lung. *J Exp Med* 2000;192:1587-1600.
130. Kuhn C, 3rd, Homer RJ, Zhu Z, Ward N, Flavell RA, Geba GP, and Elias JA. Airway hyperresponsiveness and airway obstruction in transgenic mice. Morphologic correlates in mice overexpressing interleukin (IL)-11 and IL-6 in the lung. *Am J Respir Cell Mol Biol* 2000;22:289-295.
131. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, and Elias JA. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eosinophil production. *J Clin Invest* 1999;103:779-788.
132. Zhu Z, Ma B, Zheng T, Homer RJ, Lee CG, Charo IF, Noble P, and Elias JA. IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol* 2002;168:2953-2962.
133. Weinstein M, Xu X, Ohyama K, and Deng CX. FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. *Development* 1998;125:3615-3623.
134. Yoshida M, Korfhagen TR, and Whitsett JA. Surfactant Protein D Regulates NF-kappaB and Matrix Metalloproteinase Production in Alveolar Macrophages via Oxidant-Sensitive Pathways. *J Immunol* 2001;166:7514-7519.
135. Leco KJ, Waterhouse P, Sanchez OH, Gowing KL, Poole AR, Wakeham A, Mak TW, and Khokha R. Spontaneous air space enlargement in the lungs of mice lacking tissue inhibitor of metalloproteinases-3 (TIMP-3). *J Clin Invest* 2001;108:817-829.
136. Suga T, Kurabayashi M, Sando Y, Ohyama Y, Maeno T, Maeno Y, Aizawa H, Matsumura Y, Kuwaki T, Kuro OM, Nabeshima Y, and Nagai R. Disruption of the klotho gene causes pulmonary emphysema in mice. Defect in maintenance of pulmonary integrity during postnatal life. *Am J Respir Cell Mol Biol* 2000;22:26-33.
137. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, and Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999;115:829-835.
138. Bonner JC, Rice AB, Lindroos PM, O'Brien PO, Dreher KL, Rosas I, Alfaro-Moreno E, and Osornio-Vargas AR. Induction of the lung myofibroblast PDGF receptor system by urban ambient particles from Mexico City. *Am J Respir Cell Mol Biol* 1998;19:672-680.
139. Kline JN, Cowden JD, Hunninghake GW, Schutte BC, Watt JL, Wohlford Lenane CL, Powers LS, Jones MP, and Schwartz DA. Variable airway responsiveness to inhaled lipopolysaccharide. *Am J Respir Crit Care Med* 1999;160:297-303.
140. Rylander R, Haglund P, and Lundholm M. Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom. *Am Rev Respir Dis* 1985;131:209-213.
141. Del A, Godshali MA, and Palmgren MS. Gram-negative bacterial endotoxins in grain elevator dusts. *Am Ind Hyg Assoc J* 1984;45:336-339.
142. Michel O, Kips J, Duchateau J, Vertongen F, Robert L, Collet H, Pauwels R, and Sergysels R. Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med* 1996;154:1641-1646.

143. Alexander C, and Rietschel ET. Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res* 2001;7:167-202.
144. Inohara N, Ogura Y, Chen FF, Muto A, and Nunez G. Human Nod1 confers responsiveness to bacterial lipopolysaccharides. *J Biol Chem* 2001;276:2551-2554.
145. Dentener MA. Host defense to bacterial endotoxin. Maastricht: Maastricht University; 1996.
146. Hartung J, and Seedorf J. Orienting endotoxin measurement in the atmosphere. *DTW Dtsch Tierarztl Wochenschr* 1999;106:522-525.
147. Kennedy SM, Christiani DC, Eisen EA, Wegman DH, Greaves IA, Olenchock SA, Ye TT, and Lu PL. Cotton dust and endotoxin exposure-response relationships in cotton textile workers. *Am Rev Respir Dis* 1987;135:194-200.
148. Schwartz DA, Donham KJ, Olenchock SA, Popendorf WJ, Van Fossen DS, Burmeister LF, and Merchant JA. Determinants of longitudinal changes in spirometric function among swine confinement operators and farmers. *Am J Respir Crit Care Med* 1995;151:47-53.
149. Milton DK, Wypij D, Kriebel D, Walters MD, Hammond SK, and Evans JS. Endotoxin exposure-response in a fiberglass manufacturing facility. *Am J Ind Med* 1996;29:3-13.
150. Zhiping W, Malmberg P, Larsson BM, Larsson K, Larsson L, and Saraf A. Exposure to bacteria in swine-house dust and acute inflammatory reactions in humans. *Am J Respir Crit Care Med* 1996;154:1261-1266.
151. Michel O, Nagy AM, Schroeven M, Duchateau J, Neve J, Fondu P, and Sergysels R. Dose-response relationship to inhaled endotoxin in normal subjects. *Am J Respir Crit Care Med* 1997;156:1157-1164.
152. Rylander R, Bake B, Fischer JJ, and Helander IM. Pulmonary function and symptoms after inhalation of endotoxin. *Am Rev Respir Dis* 1989;140:981-986.
153. Sandstrom T, Bjerner L, and Rylander R. Lipopolysaccharide (LPS) inhalation in healthy subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid. *Eur Respir J* 1992;5:992-996.
154. Jagielo PJ, Thorne PS, Watt JL, Frees KL, Quinn TJ, and Schwartz DA. Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects. *Chest* 1996;110:263-270.
155. Clapp WD, Becker S, Quay J, Watt JL, Thorne PS, Frees KL, Zhang X, Koren HS, Lux CR, and Schwartz DA. Grain dust-induced airflow obstruction and inflammation of the lower respiratory tract. *Am J Respir Crit Care Med* 1994;150:611-617.
156. Wesselius LJ, Nelson ME, Bailey K, and O'Brien Ladner AR. Rapid lung cytokine accumulation and neutrophil recruitment after lipopolysaccharide inhalation by cigarette smokers and nonsmokers. *J Lab Clin Med* 1997;129:106-114.
157. Schulz C, Farkas L, Wolf K, Kratzel K, Eissner G, and Pfeifer M. Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549). *Scand J Immunol* 2002;56:294-302.
158. Michel O, Duchateau J, and Sergysels R. Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. *J Appl Physiol* 1989;66:1059-1064.
159. Cavagna G, Foa V, and Vigliani EC. Effects in man and rabbits of inhalation of cotton dust or extracts and purified endotoxins. *Br J Ind Med* 1969;26:314-321.
160. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, Frees K, Watt JL, and Schwartz DA. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000;25:187-191.
161. Xing Z, Jordana M, Kirpalani H, Driscoll KE, Schall TJ, and Gauldie J. Cytokine expression by neutrophils and macrophages in vivo: endotoxin induces tumor necrosis factor- α , macrophage inflammatory protein-2, interleukin-1 β , and interleukin-6 but not RANTES or transforming growth factor- β 1 mRNA expression in acute lung inflammation [published erratum appears in *Am J Respir Cell Mol Biol* 1994 Mar;10(3):following 346]. *Am J Respir Cell Mol Biol* 1994;10:148-153.
162. Shi MM, Chong IW, Long NC, Love JA, Godleski JJ, and Paulauskis JD. Functional characterization of recombinant rat macrophage inflammatory protein-1 α and mRNA expression in pulmonary inflammation. *Inflammation* 1998;22:29-43.
163. Johnston CJ, Finkelstein JN, Gelein R, and Oberdorster G. Pulmonary cytokine and chemokine mRNA levels after inhalation of lipopolysaccharide in C57BL/6 mice. *Toxicol Sci* 1998;46:300-307.

164. Blackwell TS, Lancaster LH, Blackwell TR, Venkatakrishnan A, and Christman JW. Differential NF-kappaB activation after intratracheal endotoxin. *Am J Physiol* 1999;277:L823-830.
165. Beck-Schimmer B, Madjdpour C, Kneller S, Ziegler U, Pasch T, Wuthrich RP, Ward PA, and Schimmer RC. Role of alveolar epithelial ICAM-1 in lipopolysaccharide-induced lung inflammation. *Eur Respir J* 2002;19:1142-1150.
166. Zhang P, Bagby CJ, Kolls JK, Welsh DA, Summer WR, Andresen J, and Nelson S. The effects of granulocyte colony-stimulating factor and neutrophil recruitment on the pulmonary chemokine response to intratracheal endotoxin. *J Immunol* 2001;166:458-465.
167. Ulich TR, Watson LR, Yin SM, Guo KZ, Wang P, Thang H, and del Castillo J. The intratracheal administration of endotoxin and cytokines. I. Characterization of LPS-induced IL-1 and TNF mRNA expression and the LPS-, IL-1-, and TNF-induced inflammatory infiltrate. *Am J Pathol* 1991;138:1485-1496.
168. Ulich TR, Howard SC, Remick DG, Yi ES, Collins T, Guo K, Yin S, Keene JL, Schmuke JJ, Steininger CN, and et al. Intratracheal administration of endotoxin and cytokines: VIII. LPS induces E-selectin expression; anti-E-selectin and soluble E-selectin inhibit acute inflammation. *Inflammation* 1994;18:389-398.
169. Tang WW, Yi ES, Remick DG, Wittwer A, Yin S, Qi M, and Ulich TR. Intratracheal injection of endotoxin and cytokines. IX. Contribution of CD11a/ICAM-1 to neutrophil emigration. *Am J Physiol* 1995;269:L653-659.
170. Mizgerd JP, Scott ML, Spieker MR, and Doerschuk CM. Functions of IkappaB proteins in inflammatory responses to *Escherichia coli* LPS in mouse lungs. *Am J Respir Cell Mol Biol* 2002;27:575-582.
171. Hirano S. Quantitative time-course profiles of bronchoalveolar lavage cells following intratracheal instillation of lipopolysaccharide in mice. *Ind Health* 1997;35:353-358.
172. Wagner JG, and Roth RA. Neutrophil migration during endotoxemia. *J Leukoc Biol* 1999;66:10-24.
173. Li XY, Donaldson K, and MacNee W. Lipopolysaccharide-induced alveolar epithelial permeability: the role of nitric oxide. *Am J Respir Crit Care Med* 1998;157:1027-1033.
174. Cox G, Crossley J, and Xing Z. Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo. *Am J Respir Cell Mol Biol* 1995;12:232-237.
175. Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med* 1999;160:S5-11.
176. Yanagihara K, Seki M, and Cheng PW. Lipopolysaccharide Induces Mucus Cell Metaplasia in Mouse Lung. *Am J Respir Cell Mol Biol* 2001;24:66-73.
177. McIntosh JC, Swyers AH, Fisher JH, and Wright JR. Surfactant proteins A and D increase in response to intratracheal lipopolysaccharide. *Am J Respir Cell Mol Biol* 1996;15:509-519.
178. Sugahara K, Iyama K, Sano K, Kuroki Y, Akino T, and Matsumoto M. Overexpression of surfactant protein SP-A, SP-B, and SP-C mRNA in rat lungs with lipopolysaccharide-induced injury. *Lab Invest* 1996;74:209-220.
179. Crestani B, Rolland C, Lardeux B, Fournier T, Bernuau D, Pous C, Vissuzaine C, Li L, and Aubier M. Inducible expression of the alpha1-acid glycoprotein by rat and human type II alveolar epithelial cells. *J Immunol* 1998;160:4596-4605.
180. Klein RD, Su GL, Aminlari A, Alarcon WH, and Wang SC. Pulmonary LPS-binding protein (LBP) upregulation following LPS-mediated injury. *J Surg Res* 1998;78:42-47.
181. Fehrenbach H. Alveolar epithelial type II cell: defender of the alveolus revisited. *Respir Res* 2001;2:33-46.
182. Stolk J, Rudolphus A, Davies P, Osinga D, Dijkman JH, Agarwal L, Keenan KP, Fletcher D, and Kramps JA. Induction of emphysema and bronchial mucus cell hyperplasia by intratracheal instillation of lipopolysaccharide in the hamster. *J Pathol* 1992;167:349-356.
183. Rudolphus A, Stolk J, van Twisk C, van Noorden CJ, Dijkman JH, and Kramps JA. Detection of extracellular neutrophil elastase in hamster lungs after intratracheal instillation of *E. coli* lipopolysaccharide using a fluorogenic, elastase-specific, synthetic substrate. *Am J Pathol* 1992;141:153-160.
184. Rudolphus A, Stolk J, Dijkman JH, and Kramps JA. Inhibition of lipopolysaccharide-induced pulmonary emphysema by intratracheally instilled recombinant secretory leukocyte proteinase inhibitor. *Am Rev Respir Dis* 1993;147:442-447.

185. Stolk J, Heinzel Wieland R, Saunders D, Dijkman JH, and Steffens G. Potency of an oxidation-resistant mutant of secretory leukocyte proteinase inhibitor in lipopolysaccharide-induced emphysema in hamsters. *Pulm Pharmacol* 1993;6:33-39.
186. Stolk J, Rossie W, and Dijkman JH. Apocynin improves the efficacy of secretory leukocyte protease inhibitor in experimental emphysema. *Am J Respir Crit Care Med* 1994;150:1628-1631.
187. Toward TJ, and Broadley KJ. Goblet cell hyperplasia, airway function, and leukocyte infiltration after chronic lipopolysaccharide exposure in conscious Guinea pigs: effects of rolipram and dexamethasone. *J Pharmacol Exp Ther* 2002;302:814-821.
188. Toward TJ, and Broadley KJ. Airway reactivity, inflammatory cell influx and nitric oxide in guinea-pig airways after lipopolysaccharide inhalation. *Br J Pharmacol* 2000;131:271-281.
189. Toward TJ, and Broadley KJ. Chronic lipopolysaccharide exposure on airway function, cell infiltration, and nitric oxide generation in conscious guinea pigs: effect of rolipram and dexamethasone. *J Pharmacol Exp Ther* 2001;298:298-306.
190. Harkema JR, and Hotchkiss JA. Ozone- and endotoxin-induced mucous cell metaplasias in rat airway epithelium: novel animal models to study toxicant-induced epithelial transformation in airways. *Toxicol Lett* 1993;68:251-263.
191. Corbel M, Theret N, Caulet-Maugendre S, Germain N, Lagente V, Clement B, and Boichot E. Repeated endotoxin exposure induces interstitial fibrosis associated with enhanced gelatinase (MMP-2 and MMP-9) activity. *Inflamm Res* 2001;50:129-135.
192. Savov JD, Gavett SH, Brass DM, Costa DL, and Schwartz DA. Neutrophils play a critical role in development of LPS-induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L952-962.
193. George CL, Jin H, Wohlford-Lenane CL, O'Neill ME, Phipps JC, O'Shaughnessy P, Kline JN, Thorne PS, and Schwartz DA. Endotoxin responsiveness and subchronic grain dust-induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L203-213.

CHAPTER 2

Aims and outline of the thesis

Until a decade ago, little effort has been put into investigating the molecular and cell biology of COPD and into development of new treatments. COPD is, however, a disease with a worldwide rise in incidence and a large consumption of health care costs. The last years, COPD has received more attention leading to an increase in the knowledge on COPD. Studies on chronic local inflammatory response in patients with COPD demonstrated a specific cellular pattern consisting of predominantly macrophages, neutrophils, and CD8+ T-lymphocytes that, however, varies in the different compartments of the lung. Cytokines and chemokines were shown to be involved in recruitment of these inflammatory cells. In addition, members of the MMP family were suggested to play a role in the progression of emphysema, although a causal relationship between MMP activity and tissue destruction has as yet not been demonstrated. Furthermore, there is increasing evidence of systemic (i.e. extrapulmonary) inflammation in patients with COPD, which is considered to have an important negative influence on the quality of life. At present, however, information regarding the role of chronic inflammation – both local and systemic – in the pathogenesis of COPD is still limited and fragmentary.

Recent studies indicate that LPS may have a larger role in the chronic inflammatory process in COPD than previously realized. LPS is major constituent of Gram-negative bacteria, with strong pro-inflammatory capacities both *in vitro* and *in vivo*. The respiratory system of patients with COPD is continuously exposed to LPS due to chronic inhalation of airborne particles like cigarette smoke, organic dusts, and air pollution (indoor-outdoor) as well as aspiration of bacteria in case of chronic infection. However, inflammatory and pathologic effects of acute and chronic LPS exposure to the lung are currently not fully investigated.

Therefore, the aims of the present thesis were:

- To investigate the relationship between local and systemic inflammation in COPD patients, and to further characterize the local inflammatory response;
- To study the effects of acute and long-term LPS exposure to the lung *in vivo* using an experimental animal model.

In Chapter 3 and 4, we investigated the relationship between the local inflammatory response in the respiratory organ and systemic inflammation in patients with mild-to-moderate COPD. We assessed airway inflammation using

induction of sputum by inhalation of hypertonic saline, which is a safe, non-invasive, and reproducible method to determine cell counts and protein concentrations in the airway compartment of patients with COPD. In Chapter 3, levels of sTNF-receptors, TNF- α , and IL-8 in induced sputum and plasma were compared between COPD patients and healthy smokers matched for age and smoking history. Furthermore, persistence of the local and systemic inflammatory response was assessed by comparing current smokers and ex-smokers. In Chapter 4, levels of three major acute-phase reactants – AGP, CRP, and LBP – in induced sputum and the circulation were compared between COPD patients and healthy smokers.

In Chapter 5 and 6, we further characterized the local inflammatory response in patients with mild-to-moderate COPD. In Chapter 5, we assessed sputum levels of leptin and focussed on the role of leptin as proinflammatory mediator in the local inflammatory response in COPD. In Chapter 6, we investigated activity of three major matrix metalloproteinases – MMP-1, MMP-8, and MMP-9 – in induced sputum of COPD patients.

In Chapter 7 and 8, we assessed effects of acute intratracheal LPS instillation using a well-characterized murine model, and emphasis was placed on airway injury as well as acute-phase response induced by local LPS exposure. In Chapter 7, apoptotic cell death in bronchial epithelial cells was assessed and the role of infiltrating neutrophils and TNF- α in LPS-induced apoptosis was investigated. In Chapter 8, the effects of local LPS exposure on both pulmonary and hepatic expression of four major acute-phase reactants – SAP, AGP, LBP, and α_1 -AT – were assessed.

In Chapter 9, the inflammatory and pathological effects of long-term LPS exposure to the lung were investigated in detail. To this end, a murine model in which mice were exposed to repeated intratracheal instillation of *E.coli* LPS was developed.

Chapter 10 comprises the general discussion of the thesis and gives implications for future investigations.

CHAPTER 3

Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble TNF-receptors are increased in sputum

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is characterized by significant chronic inflammation in the pulmonary compartment as well as in the circulation. This study aimed to elucidate the relationship between local and systemic inflammation in smoking-induced COPD by assessing levels of soluble (s) tumor necrosis factor (TNF) receptors, TNF- α , and interleukin-8 (IL-8) in induced sputum and in plasma. Sputum induction was performed in 18 subjects with COPD (FEV₁ 56% predicted) and 17 healthy smokers (FEV₁ 99% predicted). Patients with COPD showed significantly higher percentages of neutrophils and levels of sTNF-R55 and IL-8 in sputum as compared with control subjects, whereas sputum sTNF-R75 levels tended to be higher in COPD. Sputum TNF- α levels were similar in both groups. When comparing sTNF receptors in sputum and plasma, no direct correlations were found despite elevation of circulating sTNF-R75 levels in patients with COPD. In addition, sputum sTNF receptors were inversely related to the FEV₁ in patients with COPD, whereas circulating sTNF receptors were not, suggesting different regulation of inflammation in the pulmonary and systemic compartment. When subjects were divided according to their current smoking status, levels of sTNF-R55, sTNF-R75, and IL-8 in sputum were significantly elevated in ex-smoking versus currently smoking patients with COPD, suggesting ongoing inflammation in airways and circulation of patients with COPD after smoking cessation.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by the progressive development of airflow limitation that is not fully reversible (reviewed by Barnes¹). The main risk factor for COPD is cigarette smoking, and at present, cessation of smoking is the only intervention with a significant attenuation of the degree of lung function impairment²⁻⁴. There is increasing evidence that COPD is associated with a chronic inflammatory response in both airways and lung parenchyma. Histopathologic studies have shown that patients with COPD have increased numbers of macrophages and CD8+ T cells in the peripheral airways and lung parenchyma⁵⁻⁸, which may be associated with latent adenoviral infection^{9,10}. On the other hand, a marked increase in neutrophils in sputum and bronchoalveolar lavage fluid was demonstrated in patients with COPD compared with control subjects^{11,12}, reflecting neutrophilia in the central airways¹³. The extent of neutrophilia correlated with the degree of airflow limitation^{12,14,15}. Analysis of induced sputum revealed that levels of interleukin-8 (IL-8) and tumor necrosis factor- α (TNF- α), which are generally considered to be important mediators in neutrophil recruitment, are elevated in patients with COPD^{14,16,17}. In addition, elevated levels of neutrophil granule proteins were demonstrated in induced sputum and bronchoalveolar lavage fluid from patients with severe COPD and smoking subjects with subclinical emphysema, respectively, indicating activation of recruited neutrophils^{18,19}.

TNF- α is a potent proinflammatory cytokine known to exert its activities by interaction with two structurally related, but functionally distinct, transmembrane receptors, referred to as TNF-R55 and TNF-R75 in accordance with their molecular weight (reviewed by Aderka²⁰). Although the two receptors are independently coexpressed on the surface of most cell types, several studies have shown that TNF-R55 is mainly expressed on cells of epithelial origin, whereas TNF-R75 is primarily found on the cell surface of cells of myeloid origin^{21,22}. A variety of inflammatory stimuli, including endogenous TNF- α formation²³, is known to induce proteolytic shedding of the extracellular cytokine-binding domains of the TNF receptors. Therefore, soluble TNF (sTNF) receptors are often considered as markers of a proinflammatory state.

Besides the presence of chronic local inflammation in the respiratory organ, there is increasing evidence of systemic inflammation in patients with stable disease as well as during episodes of acute exacerbations. Recent studies demonstrated that levels of sTNF-R55 and sTNF-R75 were significantly increased in the circulation of patients with COPD²⁴⁻²⁸. The presence of a systemic inflammatory response has an important influence on the quality of life as weight loss and muscle wasting are linked to systemic inflammation^{24,28-30}. The main cause for the presence of systemic inflammation in patients with COPD still remains to be elucidated. Systemic hypoxia is suggested to be a good candidate, as systemic hypoxia is associated with activation of the TNF- α system in patients with COPD²⁷. However, no information is yet available comparing the local inflammatory involvement in the respiratory organ with the presence of a systemic

inflammatory response in COPD. In addition, except for elevation of TNF- α in sputum of patients with COPD, no information is currently available about sTNF receptors in sputum of patients with COPD. Therefore, this study is directed at unraveling the relationship between local and systemic inflammation in COPD. To this end, levels of sTNF receptors, TNF- α , and IL-8 in induced sputum and plasma were compared. Furthermore, persistence of the local and systemic inflammatory response after smoking cessation was assessed. To this end, subjects were divided according to their current smoking status, and levels of inflammatory mediators in induced sputum and plasma were compared between current smokers and ex-smoking individuals who completely abstained for at least 1 year.

MATERIAL & METHODS

Subjects

Eighteen patients with smoking-related, clinically stable COPD followed up by general practitioners participated in the study. All of the patients met American Thoracic Society criteria for the diagnosis of COPD³¹. Inclusion criteria for patients with COPD included stable airflow limitation with an FEV₁ of less than 70% predicted with reversibility of less than 11% predicted FEV₁ or less than 200 ml after inhaled β -agonist administration and a previous history of at least 20 pack-years of smoking. Six subjects with COPD were ex-smokers who had quit smoking for at least 1 year before the start of the study. Regular use of inhaled steroids was considered as an exclusion criterion for participation to the study: none of the studied patients with COPD therefore used inhaled steroids. Thirteen patients were prescribed combination therapy (fenoterol/ipratropiumbromide) on a regular basis. Two patients were on long-acting β 2-agonists, and all other patients were prescribed bronchodilating agents on demand. Furthermore, a history of respiratory diseases other than COPD as well as increased respiratory complaints or respiratory tract infection during 4 weeks preceding the study was considered a criterion for exclusion. A control group of so-called healthy smokers was recruited that consisted of 17 subjects with a normal FEV₁ and no medical history of lung disease. A smoking history of at least 15 pack-years was used as criterion for inclusion. Seven control subjects were ex-smokers. The control subjects lived in the same geographic area as the patient population. The study was approved by the medical ethics committee of the University Hospital of Maastricht. Written informed consent was obtained from all subjects.

Pulmonary function test and blood sampling

FEV₁ was measured by trained lung function technicians using a spirometer (Masterlab; Jaeger, Würzburg, Germany) before and 15 minutes after inhalation of β -agonist via a metered-dose inhaler. Blood samples were collected in evacuated blood collecting tubes containing ethylenediaminetetraacetic acid (EDTA; Sherwood Medical, St. Louis, MO) before sputum induction (8:00 to 10:00 A.M.). Plasma samples were stored at -80°C until analyzed.

Table 1. Characteristics of the study groups investigated*

	COPD (n=18)	Healthy Smokers (n=17)	p Value ¹
Mean age, yr	60.9 ± 8.1	54.6 ± 6.9	0.017
Gender, M/F	16/2	6/11	0.001
Mean FEV ₁ , % predicted	55.5 ± 14.3	98.7 ± 16.2	< 0.001
Pack-years	47.9 ± 28.0	32.3 ± 13.2	0.045
Current smoker	12	10	NS ²
Ex-smoker	6	7	NS ²

* Values are means ± SD; ¹ Student T-test; ² Chi-square test. COPD = chronic obstructive pulmonary disease; M = male; F = female; FEV₁ = forced expiratory volume in 1 second; pack-year = smoking 1 pack per day for 1 year; ex-smoker = quitting smoking for at least 1 year before the start of the study; NS = not significant.

Sputum induction and processing

Sputum was induced after a procedure as reported elsewhere¹⁴. Briefly, subjects inhaled 3% hypertonic saline, nebulized via an ultrasonic nebulizer (NEB2000; TEFA-Portanje, Woerden, The Netherlands) during three 7-minute periods. Before expectoration, subjects were instructed to mouthwash thoroughly with saline solution to minimize saliva contamination. The collected sputum was pooled and kept at 4°C for not more than 2 hours before further processing.

The volume of the pooled sputum (without selection of sputum plugs) was recorded, and an equal volume of 0.2% dithiothreitol (DTT; Sputolysin; Calbiochem, La Jolla, CA) was added. The samples were then mixed gently by a vortex mixer and incubated for 20 minutes at room temperature to ensure complete homogenization. Cell-free supernatants were frozen at -80°C until subsequent analysis. The total cell count and cell viability were assessed using a standard hemocytometer (Coulter Z1; Coulter Electronics, Mijdrecht, The Netherlands) and by trypan blue exclusion, respectively. Cytospins were stained according to the May-Grünwald Giemsa method. Differential cell counts were performed by one observer blinded to the clinical characteristics, counting 500 nucleated cells. The numbers of squamous epithelial cells were subtracted, and the differential cell counts were expressed as corrected percentages. A sample was considered adequate if the slides contained 15% or less squamous epithelial cells.

Table 2. Total and differential cell counts in patients with COPD and healthy smokers*

	COPD (n=18)	Healthy Smokers (n=17)	p Value ¹
Total cell count, 10 ⁶ cells/ml	4.0 ± 3.3	4.0 ± 4.1	NS
Viability, %	90.3 ± 7.8	90.3 ± 10.0	NS
AM, %	17.1 ± 10.9	36.1 ± 15.9	< 0.001
PMN, %	80.5 ± 12.4	62.4 ± 16.4	0.001
Eos, %	1.8 ± 3.6	0.7 ± 1.0	NS
Lym, %	0.7 ± 1.2	0.9 ± 0.8	NS

* Data are presented as means ± SD; ¹ ANOVA adjusted for age, gender, and pack-years. COPD = chronic obstructive pulmonary disease; AM = alveolar macrophages; PMN = neutrophils; Eos = eosinophils; Lym = lymphocytes; NS = not significant.

Analysis of sTNF receptors, TNF- α , and IL-8 levels in sputum and plasma

sTNF-R55 and sTNF-R75 assays. sTNF-R were measured in sputum supernatant and plasma using specific sandwich enzyme-linked immunosorbent assay (ELISA) as described^{32,33}. Rabbit anti-human sTNF-R55 polyclonal antibody and murine anti-human sTNF-R75 monoclonal antibody (MR2-2) were used as coating antibodies. Assays for sTNF-R were not affected by presence of TNF α ³³, indicating that total amounts of sTNF-R55 and sTNF-R75 (free and bound to TNF- α) were measured. The lower detection limit was 60 pg/ml for sTNF-R55 and 30 pg/ml for sTNF-R75.

TNF- α assays. Levels of TNF- α were measured using (1) a specific sandwich ELISA as described previously^{34,35} that recognizes bioactive TNF- α with a lower detection limit of 15 pg/ml as well as by (2) a commercially available ELISA (HyCult Biotechnology BV, Uden, The Netherlands) that recognizes free TNF- α as well as TNF- α bound to sTNF receptors (referred to as total TNF- α fraction; personal communication HyCult Biotechnology BV) with a lower limit of detection of 20 pg/ml.

IL-8 assay. IL-8 levels were determined using specific sandwich ELISA as described by Bouma and colleagues³⁶. The lower detection limit was 8 pg/ml.

DTT effect. To study the effect of DTT on ELISA determination of the inflammatory mediators assessed, standard curves of appropriate recombinant proteins were incubated with 0.1% DTT (Sputolysin) under the same conditions as sputum samples during processing (20 minutes at room temperature). The presence of DTT resulted in less than 5% inhibition of the detection of sTNF-R55 and IL-8 by ELISA and less than 10% inhibition in case of sTNF-R75, bioactive TNF- α , and total TNF- α fraction, indicating that DTT has little or no effect on the assays used in this study (data not shown).

Microvascular leakage

Microvascular leakage was determined in each individual as follows: sputum albumin (mg/L)/serum albumin (g/L). The albumin concentration in sputum and plasma was measured using a newly developed ELISA. Immunomaxisorp plates (Nunc, Roskilde, Denmark) were coated overnight with albumin-specific monoclonal antibody 4D5 (HM17). Diluted plasma samples and a standard dilution series with human albumin (Sigma, St. Louis, MO) were added to the plate. Albumin was detected with a horseradish peroxidase-labeled polyclonal rabbit anti-human albumin immunoglobulin G (Cappel; Organon Teknika Corporation, West Chester, PA) followed by 3',5,5'-tetramethylbenzidine (Kirkegaard and Perry Lab, Gaithersburg, MD) as substrate. The lower detection limit was 10 ng/ml. The presence of DTT resulted in a less than 5% inhibition of the detection of albumin by ELISA.

Statistical analysis

Results are presented as mean $\pm$ SD for normally distributed variables and median (range) otherwise. Groups were compared by analysis of covariance adjusted for age, sex, or pack-years where appropriate. Nonparametric data were compared by the Mann-Whitney U test. The chi-square test was used to compare

categorical variables. Correlations between parameters were evaluated using Pearson's rank correlation analysis (Statistical Package for the Social Sciences, version 10.0 for Windows; SPSS Inc., Chicago, IL). A p value of less than 0.05 denotes the presence of a significant statistical difference.

RESULTS

Clinical characteristics

Clinical characteristics of patients with COPD and healthy smokers are shown in Table 1. Patients with COPD had significant airflow limitation, whereas control subjects had normal FEV₁ values (percentage predicted) according to the selection criteria.

Sputum characteristics

All subjects included in this study tolerated well the procedure of sputum induction, and an adequate specimen of sputum was collected in every participant. Induced sputum was analyzed for total and differential cell counts, as shown in Table 2. The total cell counts and cell viability in sputum were not different in patients with COPD versus healthy smokers. Neutrophils (percentage) were significantly higher in patients with COPD in comparison with control subjects, whereas macrophages (percentage) were significantly lower.

Table 3. Inflammatory mediators in sputum of patients with COPD and healthy smokers*

	COPD (n=18)	Healthy Smokers (n=17)	p Value [†]
IL-8, ng/ml	3.7 (1.4-12.2)	2.3 (0.8-5.2)	0.008
Bioactive TNF- α , pg/ml	<15 [‡]	<15 [‡]	NS
Total TNF- α fraction, pg/ml	59 (20-143)	57 (20-206)	NS
sTNF-R55, pg/ml	237 (60-2,501)	109 (60-435)	0.020
sTNF-R75, pg/ml	70 (30-911)	77 (30-256)	NS

* Data are presented as median (range); [†] Mann-Whitney U test; [‡] Below the detection limit. COPD = chronic obstructive pulmonary disease; IL-8 = interleukin-8; TNF- α = tumor necrosis factor- α ; NS = not significant; sTNF-R55 = soluble TNF receptor 55; sTNF-R75 = soluble TNF receptor 75.

In addition, levels of the neutrophil chemoattractants IL-8 and TNF- α were determined in the sputum supernatant. IL-8 levels were significantly elevated in patients with COPD compared with healthy smokers (Table 3) and were strongly correlated with the number of neutrophils in the COPD group, but not in the control group (Table 4). Concerning TNF- α , both bioactive TNF- α and the total TNF- α fraction (free TNF- α and TNF- α bound to sTNF receptors) were determined (Table 3). Bioactive TNF- α was not detected in sputum samples of either patients with COPD or healthy smokers. Using the assay for the total TNF- α fraction, low concentrations of TNF- α were detected, but total TNF- α in sputum was not significantly different between the two groups.

Table 4. Correlations between sputum markers in patients with COPD and healthy smokers*

	COPD (n = 18)		Healthy Smokers (n = 17)	
	R Value	p Value	R Value	p Value
IL-8/neutrophils	0.704	0.001	0.345	NS
sTNF-R55/sTNF-R75	0.973	<0.001	0.750	0.001
sTNF-R55/neutrophils	0.677	0.002	0.414	NS
sTNF-R75/neutrophils	0.607	0.008	0.362	NS
sTNF-R55/IL-8	0.699	0.001	0.742	<0.001
sTNF-R75/IL-8	0.562	0.015	0.780	<0.001
sTNF-R55/total TNF- α	0.177	NS	-0.105	NS
sTNF-R75/total TNF- α	0.171	NS	0.032	NS

* Pearson's rank correlation. COPD = chronic obstructive pulmonary disease; IL-8 = interleukin-8; sTNF-R55 = soluble TNF receptor 55; sTNF-R75 = soluble TNF receptor 75; TNF- α = tumor necrosis factor- α ; NS = not significant.

To characterize further the local inflammation in COPD, sputum levels of sTNF-R55 and sTNF-R75 were determined. As shown in Table 3, both sTNF receptors were detectable in induced sputum of patients with COPD and healthy smokers. Sputum sTNF-R55 levels in patients with COPD patients showed a twofold increase as compared with healthy smokers. Sputum sTNF-R75 levels showed a tendency to increase in patients with COPD, but did not reach the level of statistical significance ($p = 0.08$). As shown in Table 4, sputum levels of sTNF-R55 and sTNF-R75 correlated highly with each other, and with IL-8 levels in both study groups. In contrast, sputum levels of the sTNF receptors strongly correlated with the percentage of neutrophils in patients with COPD, but this was not the case in healthy smokers. To address discrepancy, study groups were split according to the level of sputum neutrophils using the mean + 1 x SD of sputum neutrophils in healthy controls as cutoff point. As shown in Figure 1, neutrophils (percentage) in sputum of 11 patients with COPD and 2 healthy smokers were out of the range of those found in healthy smokers. Furthermore, there was a large variance in sputum levels of sTNF-R55 and sTNF-R75 in patients with COPD with high neutrophil levels, whereas this was not the case in patients with COPD with low neutrophil levels.

Figure 1. Levels of (A) sTNF-R55 and (B) sTNF-R75 in induced sputum obtained from patients with COPD and healthy smokers (HS). Study groups were split according to the level of sputum neutrophils using the mean + 1xSD of sputum neutrophils in healthy controls as cut-off point (78.8%). The open circle symbolizes outliers.

Next, we investigated whether the pulmonary inflammation was related to the degree of airflow limitation. To this end, we looked for correlations between the FEV_1 (percentage predicted) and sputum levels of IL-8, sTNF receptors, and TNF- α . As shown in Figure 2, sputum levels of IL-8 and sTNF-R55 strongly correlated with airflow limitation in patients with COPD but not in healthy smokers. Sputum sTNF-R75 levels tended to correlate with the FEV_1 in patients with COPD, but this did not reach the level of statistical significance (sTNF-R75, $r = -0.444$, $p = 0.08$). The total TNF- α in sputum was not related to airflow limitation in patients with COPD ($r = -0.258$, $p = 0.317$).

Figure 2. Correlation between the forced expiratory volume in 1 second (FEV_1) and sputum levels of (A) IL-8 (COPD: $r = -0.836$, $p < 0.001$; control: $r = 0.093$, $p = 0.723$) or (B) sTNF-R55 (COPD: $r = 0.519$, $p = 0.033$; control: $r = 0.213$, $p = 0.411$) in patients with COPD (closed diamonds) and healthy smokers (open diamonds).

Plasma levels of sTNF receptors, TNF- α , and IL-8

Systemic inflammation was assessed by determination of plasma levels of sTNF-R55, sTNF-R75, TNF- α , and IL-8 by specific ELISA. As shown in Table 5, no significant difference was seen in circulating sTNF-R55 levels of patients with COPD and healthy control subjects. In contrast, plasma levels of sTNF-R75 were significantly increased in patients with COPD. Like in sputum, plasma levels of sTNF-R55 and sTNF-R75 correlated highly with each other in both patients with COPD ($r = 0.797$, $p < 0.001$) and healthy smokers ($r = 0.732$, $p = 0.001$). IL-8 was detectable in 4 out of 18 patients with COPD in comparison with none of the 17 control subjects, resulting in significantly increased levels of IL-8 in the COPD group. Circulating TNF- α was measured using the assay for total TNF- α . Plasma TNF- α levels of all individuals except two were below the lower detection limit of the assay.

In addition, we investigated whether the systemic inflammation was related to the degree of airflow limitation by analyzing correlations between the FEV_1 (percentage predicted) and plasma levels of IL-8 and sTNF receptors. In contrast to inflammatory mediators in sputum, circulating levels of the sTNF receptors and IL-8 did not show any correlation with the degree of airflow limitation (data not shown).

Table 5. Inflammatory mediators in plasma of patients with COPD and healthy smokers*

		COPD (n=18)		Healthy Smokers (n=17)	p Value [†]
sTNF-R55, ng/ml		2.0 (1.4-3.3)		1.7 (1.2-2.6)	NS
sTNF-R75, ng/ml		1.9 (1.3-2.9)		1.4 (1.1-2.7)	0.004
Total TNF- α , pg/ml	n = 17	<20 [‡]	n = 15	<20 [‡]	NS
	n = 1	36.6	n = 1	34.9	
IL-8, pg/ml	n = 14	<8.0 [‡]	n = 16	<8.0 [‡]	0.049
	n = 4	9.9 (8.4-10.6)			

* Data are presented as median (range); [†] Mann-Whitney U test; [‡] Below the detection limit. COPD = chronic obstructive pulmonary disease; IL-8 = interleukin-8; NS = not significant; sTNF-R55 = soluble TNF receptor 55; sTNF-R75 = soluble TNF receptor 75; TNF- α = tumor necrosis factor- α .

Relationship between local and systemic compartment

To investigate the possible relationship between local and systemic inflammation in patients with COPD, we first determined to what extent the data are affected by microvascular leakage. To this end, we measured albumin levels in sputum and plasma of patients with COPD and healthy smokers and calculated the level of microvascular leakage in each individual as follows: sputum albumin (mg/L)/serum albumin (g/L). Sputum-to-plasma ratios of albumin were not significantly different between the two study groups (median [range] COPD, 1.68 [0.41–6.11]; control, 1.31 [0.29–4.00], $p > 0.05$), indicating a similar degree of microvascular leakage in patients with COPD and healthy smokers. Therefore, the values of the inflammatory mediators in sputum were not corrected for microvascular leakage.

Next, we looked for correlations between sputum and plasma levels of sTNF-R55, sTNF-R75, and IL-8 in patients with COPD and healthy smokers to examine the relationship between local and systemic inflammation. As shown in Table 6, no direct correlations were found between sputum and plasma concentrations of each inflammatory mediator analyzed in either patients with COPD or in healthy smokers.

Table 6. Correlations between sputum and plasma levels of inflammatory mediators in patients with COPD and healthy smokers*

Sputum to plasma	COPD (n=18)		Healthy Smokers (n=16)	
	R Value	p Value	R Value	p Value
sTNF-R55	0.155	0.539	0.359	0.172
sTNF-R75	0.068	0.788	0.377	0.150
IL-8	0.292	0.240	-	-

* Pearson's rank correlation. COPD = chronic obstructive pulmonary disease; IL-8 = interleukin-8; NS = not significant; sTNF-R55 = soluble TNF receptor 55; sTNF-R75 = soluble TNF receptor 75; TNF = tumor necrosis factor.

Local and systemic inflammation in current smokers versus ex-smokers

To investigate the effect of smoking cessation on the local and systemic level of inflammation, subjects in both groups were divided according to their current smoking status, and levels of inflammatory mediators were compared between current and ex-smoking individuals. As shown in Table 7, sputum levels of sTNF-R55, sTNF-R75, and IL-8 were not different between current and ex-smokers in

the control group. On the other hand, sputum levels of sTNF-R55, sTNF-R75, and IL-8 were significantly increased in patients with COPD who are ex-smokers compared with currently smoking patients with COPD. In addition, sTNF-R55 and IL-8 levels in sputum from ex-smoking patients with COPD were also significantly raised in comparison with ex-smoking healthy subjects, whereas sTNF-R75 levels showed a tendency to increase in ex-smoking patients with COPD ($p = 0.09$). In contrast, circulating levels of all inflammatory mediators assessed were not different in current smokers compared with ex-smokers in both study groups.

Table 7. Levels of soluble TNF-R55, TNF-R75, and IL-8 in current smokers versus ex-smokers*

	COPD		Healthy Smokers	
	Ex-smokers (n=6)	Current smokers (n=12)	Ex-smokers (n=7)	Current smokers (n=10)
Sputum				
sTNF-R55, pg/ml	1,097 (60-2,026) [†]	389 (60-2,501)	213 (60-435)	139 (60-428)
sTNF-R75, pg/ml	365 (52-782) [†]	138 (30-911)	110 (30-256)	67 (30-190)
IL-8, ng/ml	8.3 (2.3-12.2) [†]	3.8 (1.4-11.9)	2.9 (1.0-4.1)	2.1 (0.8-5.2)
Plasma				
sTNF-R55, ng/ml	2.0 (1.6-2.4)	2.0 (1.4-3.3)	1.7 (1.2-2.4)	1.9 (1.2-2.6)
sTNF-R75, ng/ml	2.0 (1.3-2.7)	1.9 (1.4-2.9)	1.6 (1.1-2.7)	1.5 (1.1-2.0)
IL-8, pg/ml	n=3, 9.7 (8.4-10.4)	n=1, 10.6	ND	ND

* Data are presented as median (range); [†] $p < 0.05$ compared with current smoking COPD patients (Mann-Whitney U test); [‡] $p < 0.05$ compared with ex-smoking control subjects (Mann-Whitney U test). COPD = chronic obstructive pulmonary disease; IL-8 = interleukin-8; ND = not detectable; sTNF-R55 = soluble TNF receptor 55; sTNF-R75 = soluble TNF receptor 75; TNF = tumor necrosis factor.

DISCUSSION

This study focused on the relationship between local and systemic inflammation in patients with mild-to-moderate smoking-related COPD. Analysis of induced sputum showed a significant increase in neutrophils (percentage) and IL-8 levels in patients with COPD versus healthy smokers, which correlated highly with each other and with the degree of airflow limitation. These findings are in line with previous observations in patients with COPD^{11,14,16,17}. Concerning TNF- α , we found no significant differences in sputum TNF- α levels between mild-to-moderate patients with COPD and healthy smokers using either an ELISA that measures strictly intact TNF- α that is not bound by TNF receptors or using an ELISA that detects the total TNF- α fraction, that is, free TNF- α and TNF- α bound to sTNF-R. In contrast, Keatings and colleagues reported a rise in TNF- α levels in induced sputum of patients with severe COPD¹⁴. This discrepancy may be due to differences in severity of COPD in the two patient populations studied or to differences between the characteristics of the TNF- α assays used. In addition, study populations may differ in rates of bacterial colonization, which was demonstrated to result in higher TNF- α levels in spontaneously expectorated sputum from *Haemophilus influenzae*-colonized patients with COPD in comparison with noncolonized but otherwise similar patients with COPD³⁷.

Finally, neutrophil elastase that is present in neutrophil microenvironment in induced sputum of patients with COPD is a potential parameter in the degradation of TNF- α into bioinactive TNF- α ³⁸, which may hamper the detection of bioactive TNF- α in sputum.

Soluble receptors for TNF- α are considered to be markers of a proinflammatory state because of their shedding from the cell membrane in response to endogenous TNF- α and other inflammatory mediators. In this study, we showed that in particular sTNF-R55 was increased in induced sputum of patients with mild-to-moderate COPD compared with healthy smokers, which correlated with airflow limitation as well as other inflammatory markers (IL-8 and neutrophilia) in patients with COPD. Especially, respiratory epithelial cells are reported to express TNF-R55²² and may therefore account significantly for the increased levels of sTNF-R55 in sputum. In addition, *in vitro* studies by Porteu and Nathan demonstrated that activation of neutrophils resulted in shedding of both sTNF receptors from the cell surface, which suggests that sTNF-R55 and sTNF-R75 measured in sputum may also be derived from activated neutrophils that are extensively present in sputum of patients with COPD³⁹.

Previous studies from our group as well as other groups investigating systemic inflammation in patients with COPD demonstrated small but significant increases in plasma levels of sTNF-R55 and sTNF-R75 in patients with COPD in addition to enhanced levels of acute-phase proteins²⁴⁻²⁸. In line with these reports, we demonstrated that circulating sTNF-R75 levels were elevated in patients with COPD as compared with healthy smokers. The biologic significance in the sense of the role that such a difference plays in the biologic phenomenon is not clear from these studies. sTNF receptors are known to compete for TNF- α with the cell surface TNF receptors and may hereby play a role in regulating steady-state levels of TNF- α in the airways and circulation⁴⁰. Further studies using large study populations are necessary to elucidate the origin of sTNF receptors and their function – either pro-inflammatory or anti-inflammatory – in chronic inflammation in COPD.

Although it is generally accepted that systemic inflammation contributes to the impaired health status in COPD, little is known about the origin of the systemic inflammation. Therefore, we assessed the possible relationship between the local and systemic inflammatory response. First, results of this study showed that patients with COPD had increased levels of sTNF-R55 levels in sputum as compared with healthy smokers, whereas the reverse situation occurred in plasma: levels of sTNF-R75 were higher in patients with COPD than in control subjects. Second, we found no correlation between the individual inflammatory mediators in sputum compared with plasma in both study groups. Furthermore, sputum levels of sTNF-R55 and sTNF-R75 were related with degree of airflow limitation in the COPD group, whereas plasma levels of sTNF receptors were not. Taken together, these results suggest that systemic inflammation in COPD is not due to an overflow of inflammatory mediators from the local compartment but that the inflammatory responses in the local and systemic compartment are differently regulated. In line with this hypothesis, Takabatake and colleagues

recently reported that systemic hypoxia observed in patients with COPD due to deterioration of the lung function might contribute to enhanced levels of systemic inflammatory markers²⁷. Future studies are clearly required to elucidate the origin and regulation of the systemic inflammatory response in COPD.

Cigarette smoking is widely recognized as a primary risk factor for developing COPD in industrialized countries. The presence of harmful compounds in inhaled cigarette smoke, including endotoxin⁴¹, is considered to be the starting point of the pathogenetic pathway in COPD. Previous studies have shown that smoking cessation resulted in a significant reduction of the age-related decline in FEV₁²⁻⁴. However, a longitudinal follow-up study of smoking-induced lung density changes by high-resolution computed tomography demonstrated that the worsening airspace abnormality in the upper lung zones of smokers did not slow down with cessation of smoking⁴². In addition, other studies investigating airway inflammation reported that smoking cessation did not influence the cellular profile and levels of IL-8 in central and peripheral airways^{16,43-45}. In this study, we found a remarkable increase in sputum levels of sTNF-R55, sTNF-R75, and IL-8 in ex-smoking patients with COPD compared with currently smoking patients with COPD, but not in ex-smoking versus currently smoking healthy controls. In addition, sputum levels of sTNF-R55 and IL-8 in ex-smoking patients with COPD were also significantly raised in comparison with levels in ex-smoking healthy subjects. In contrast, the systemic proinflammatory state was not associated with the smoking status in both study groups. Our results are strongly supported by the recently reported data of Willemse and colleagues⁴⁶, who collected induced sputum before and 1 year after smoking cessation in smokers with COPD and without COPD. They showed that IL-8 levels in sputum decreased without changes in sputum neutrophils after 1 year of smoking cessation in smokers without COPD. In contrast, in smokers with COPD, levels of IL-8 and neutrophils in sputum tended to increase ($p < 0.1$). Taken together, these data suggest an ongoing inflammatory response in the respiratory compartment of patients with COPD after smoking cessation.

In summary, patients with COPD had significantly higher percentages of neutrophils and increased levels of sTNF-R55 and IL-8 in their induced sputum samples as compared with healthy smokers. A comparison of sTNF receptors in sputum and plasma, however, did not demonstrate direct correlations between local and systemic inflammation, despite the significantly higher circulating levels of sTNF-R75 in patients with COPD. Moreover, sputum sTNF receptors were related with the degree of airflow limitation in patients with COPD, whereas circulating sTNF receptors were not, suggesting different regulation of the inflammatory response in the local and systemic compartment. Sputum levels of sTNF-R55, sTNF-R75, and IL-8 were increased in ex-smoking versus currently smoking patients with COPD as well as ex-smoking control subjects, whereas the current smoking status was not associated with the systemic proinflammatory state in the study groups. These results confirm the presence of inflammatory processes in the airways and circulation of patients with COPD despite smoking cessation.

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REFERENCES

1. Barnes PJ. Chronic obstructive pulmonary disease. *N Engl J Med* 2000;343:269-280.
2. Anthonisen NR, Connett JE, Kiley JP, Altose MD, Bailey WC, Buist AS, Conway WA, Jr., Enright PL, Kanner RE, O'Hara P, and et al. Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV1. The Lung Health Study. *Jama* 1994;272:1497-1505.
3. Scanlon PD, Connett JE, Waller LA, Altose MD, Bailey WC, and Buist AS. Smoking cessation and lung function in mild-to-moderate chronic obstructive pulmonary disease. The Lung Health Study. *Am J Respir Crit Care Med* 2000;161:381-390.
4. Kanner RE, Connett JE, Williams DE, and Buist AS. Effects of randomized assignment to a smoking cessation intervention and changes in smoking habits on respiratory symptoms in smokers with early chronic obstructive pulmonary disease: the Lung Health Study. *Am J Med* 1999;106:410-416.
5. Finkelstein R, Fraser RS, Ghezzi H, and Cosio MG. Alveolar inflammation and its relation to emphysema in smokers. *Am J Respir Crit Care Med* 1995;152:1666-1672.
6. Saetta M, Di Stefano A, Turato G, Facchini FM, Corbino L, Mapp CE, Maestrelli P, Ciaccia A, and Fabbri LM. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;157:822-826.
7. Saetta M, Baraldo S, Corbino L, Turato G, Braccioni F, Rea F, Cavallero G, Tropeano G, Mapp CE, Maestrelli P, Ciaccia A, and Fabbri LM. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;160:711-717.
8. Saetta M, Turato G, Baraldo S, Zanin A, Braccioni F, Mapp CE, Maestrelli P, Cavallero G, Papi A, and Fabbri LM. Goblet cell hyperplasia and epithelial inflammation in peripheral airways of smokers with both symptoms of chronic bronchitis and chronic airflow limitation. *Am J Respir Crit Care Med* 2000;161:1016-1021.
9. Retamales I, Elliott WM, Meshi B, Coxson HO, Pare PD, Sciurba FC, Rogers RM, Hayashi S, and Hogg JC. Amplification of inflammation in emphysema and its association with latent adenoviral infection. *Am J Respir Crit Care Med* 2001;164:469-473.
10. Shapiro SD. End-stage chronic obstructive pulmonary disease: the cigarette is burned out but inflammation rages on. *Am J Respir Crit Care Med* 2001;164:339-340.
11. Peleman RA, Ryttilä PH, Kips JC, Joos GF, and Pauwels RA. The cellular composition of induced sputum in chronic obstructive pulmonary disease. *Eur Respir J* 1999;13:839-843.
12. Pesci A, Balbi B, Majori M, Cacciani G, Bertacco S, Alciato P, and Donner CF. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J* 1998;12:380-386.
13. Alexis NE, Hu SC, Zeman K, Alter T, and Bennett WD. Induced Sputum Derives from the Central Airways. Confirmation using a radiolabeled aerosol bolus delivery technique. *Am J Respir Crit Care Med* 2001;164:1964-1970.
14. Keatings VM, Collins PD, Scott DM, and Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530-534.
15. Di Stefano A, Capelli A, Lusuardi M, Balbo P, Vecchio C, Maestrelli P, Mapp CE, Fabbri LM, Donner CF, and Saetta M. Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med* 1998;158:1277-1285.

16. Yamamoto C, Yoneda T, Yoshikawa M, Fu A, Tokuyama T, Tsukaguchi K, and Narita N. Airway inflammation in COPD assessed by sputum levels of interleukin-8. *Chest* 1997;112:505-510.
17. Rutgers SR, Timens W, Kaufmann HF, van der Mark TW, Koeter GH, and Postma DS. Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. *Eur Respir J* 2000;15:109-115.
18. Keatings VM, and Barnes PJ. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am J Respir Crit Care Med* 1997;155:449-453.
19. Betsuyaku T, Nishimura M, Takeyabu K, Tanino M, Venge P, Xu S, and Kawakami Y. Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Respir Crit Care Med* 1999;159:1985-1991.
20. Aderka D. The potential biological and clinical significance of the soluble tumor necrosis factor receptors. *Cytokine Growth Factor Rev* 1996;7:231-240.
21. Brockhaus M, Schoenfeld HJ, Schlaeger EJ, Hunziker W, Lesslauer W, and Loetscher H. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc Natl Acad Sci U S A* 1990;87:3127-3131.
22. Pryhuber GS, Huyck HL, Staversky RJ, Finkelstein JN, and O'Reilly MA. Tumor necrosis factor- α -induced lung cell expression of antiapoptotic genes TRAF1 and cIAP2. *Am J Respir Cell Mol Biol* 2000;22:150-156.
23. Lantz M, Malik S, Slevin ML, and Olsson I. Infusion of tumor necrosis factor (TNF) causes an increase in circulating TNF-binding protein in humans. *Cytokine* 1990;2:402-406.
24. Schols AM, Buurman WA, Staal van den Brekel AJ, Dentener MA, and Wouters EF. Evidence for a relation between metabolic derangements and increased levels of inflammatory mediators in a subgroup of patients with chronic obstructive pulmonary disease. *Thorax* 1996;51:819-824.
25. Dentener MA, Creutzberg EC, Schols AM, Mantovani A, van't Veer C, Buurman WA, and Wouters EF. Systemic anti-inflammatory mediators in COPD: increase in soluble interleukin 1 receptor II during treatment of exacerbations. *Thorax* 2001;56:721-726.
26. Takabatake N, Nakamura H, Abe S, Hino T, Saito H, Yuki H, Kato S, and Tomoike H. Circulating leptin in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;159:1215-1219.
27. Takabatake N, Nakamura H, Abe S, Inoue S, Hino T, Saito H, Yuki H, Kato S, and Tomoike H. The relationship between chronic hypoxemia and activation of the tumor necrosis factor- α system in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;161:1179-1184.
28. Eid AA, Ionescu AA, Nixon LS, Lewis-Jenkins V, Matthews SB, Griffiths TL, and Shale DJ. Inflammatory response and body composition in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164:1414-1418.
29. Di Francia M, Barbier D, Mege JL, and Orehek J. Tumor necrosis factor- α levels and weight loss in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1994;150:1453-1455.
30. De Godoy I, Donahoe M, Calhoun WJ, Mancino J, and Rogers RM. Elevated TNF- α production by peripheral blood monocytes of weight-losing COPD patients. *Am J Respir Crit Care Med* 1996;153:633-637.
31. ATS. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1995;152:S77-121.
32. Leeuwenberg JF, Dentener MA, and Buurman WA. Lipopolysaccharide LPS-mediated soluble TNF receptor release and TNF receptor expression by monocytes. Role of CD14, LPS binding protein, and bactericidal/permeability-increasing protein. *J Immunol* 1994;152:5070-5076.
33. Leeuwenberg JF, Jeunhomme TM, and Buurman WA. Slow release of soluble TNF receptors by monocytes in vitro. *J Immunol* 1994;152:4036-4043.
34. Engelberts I, Stephens S, Francot GJ, van der Linden CJ, and Buurman WA. Evidence for different effects of soluble TNF-receptors on various TNF measurements in human biological fluids. *Lancet* 1991;338:515-516.
35. Engelberts I, Moller A, Schoen GJ, van der Linden CJ, and Buurman WA. Evaluation of measurement of human TNF in plasma by ELISA. *Lymphokine Cytokine Res* 1991;10:69-76.
36. Bouma MG, Stad RK, van den Wildenberg FA, and Buurman WA. Differential regulatory effects of adenosine on cytokine release by activated human monocytes. *J Immunol* 1994;153:4159-4168.

37. Bresser P, Out TA, van Alphen L, Jansen HM, and Lutter R. Airway inflammation in nonobstructive and obstructive chronic bronchitis with chronic haemophilus influenzae airway infection. Comparison with noninfected patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;162:947-952.
38. Nortier J, Vandenabeele P, Noel E, Bosseloir Y, Goldman M, and Deschodt-Lanckman M. Enzymatic degradation of tumor necrosis factor by activated human neutrophils: role of elastase. *Life Sci* 1991;49:1879-1886.
39. Porteu F, and Nathan C. Shedding of tumor necrosis factor receptors by activated human neutrophils. *J Exp Med* 1990;172:599-607.
40. Pinckard JK, Sheehan KC, Arthur CD, and Schreiber RD. Constitutive shedding of both p55 and p75 murine TNF receptors in vivo. *J Immunol* 1997;158:3869-3873.
41. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, and Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999;115:829-835.
42. Soejima K, Yamaguchi K, Kohda E, Takeshita K, Ito Y, Mastubara H, Oguma T, Inoue T, Okubo Y, Amakawa K, Tateno H, and Shiomi T. Longitudinal follow-up study of smoking-induced lung density changes by high-resolution computed tomography. *Am J Respir Crit Care Med* 2000;161:1264-1273.
43. Mullen JB, Wright JL, Wiggs BR, Pare PD, and Hogg JC. Structure of central airways in current smokers and ex-smokers with and without mucus hypersecretion: relationship to lung function. *Thorax* 1987;42:843-848.
44. Turato G, Di Stefano A, Maestrelli P, Mapp CE, Ruggieri MP, Roggeri A, Fabbri LM, and Saetta M. Effect of smoking cessation on airway inflammation in chronic bronchitis. *Am J Respir Crit Care Med* 1995;152:1262-1267.
45. Rutgers SR, Postma DS, ten Hacken NH, Kauffman HF, van Der Mark TW, Koeter GH, and Timens W. Ongoing airway inflammation in patients with COPD who do not currently smoke. *Thorax* 2000;55:12-18.
46. Willemse BW, Ten Hacken NH, Rutgers B, G. Li, Postma DS, and Timens W. The effect of one year smoking cessation on neutrophilic inflammation in smokers with and without COPD. *Am J Respir Crit Care Med* 2002;165:A463.

CHAPTER 4

Local and systemic acute-phase response in COPD

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ABSTRACT

Introduction: Acute-phase reactants (APRs) play an important role in innate immunity. As expression of APRs in the airways may enhance local response to injury and infection in COPD, this study aimed to assess levels of α_1 -acid glycoprotein (AGP), lipopolysaccharide-binding protein (LBP), and C-reactive protein (CRP) in induced sputum of COPD patients and healthy smokers. *Methods:* Sputum induction was performed in 18 patients with COPD (FEV₁ 58% predicted) and 17 healthy smokers (FEV₁ 99% predicted). AGP, LBP, and CRP were analyzed in sputum supernatant and plasma by ELISA. *Results:* AGP, LBP, and CRP were present in sputum of all subjects. Sputum levels of AGP and LBP were significantly increased in COPD patients versus healthy smokers, and inversely correlated with the FEV₁ values in COPD. In contrast, sputum CRP was not different between the study groups. In plasma, LBP was markedly elevated in COPD patients versus healthy smokers, whereas circulating levels of AGP and CRP tended to be increased in COPD. In contrast to sputum, AGP and LBP in plasma were positively related to the FEV₁ values. When APRs in sputum and plasma were compared, no direct relations were found despite that APRs were strongly related with each other in each compartment. *Conclusion:* AGP and LBP were significantly increased in sputum of COPD patients, which may be due to local production rather than leakage from the systemic compartment.

INTRODUCTION

The acute-phase response is a coordinated series of physiological and biochemical changes executed by the host, and thought to be beneficial with the aim of restoring the disturbed physiological homeostasis and preventing ongoing tissue damage. Substantial changes in plasma acute-phase reactants (APRs) are commonly observed during acute infection, trauma, surgery, and burns. On the other hand, small but significant elevations in systemic APRs are strongly associated with chronic inflammatory disorders. For example, we and others have demonstrated that patients suffering from chronic obstructive pulmonary disease (COPD) have increased plasma levels of C-reactive protein (CRP), lipopolysaccharide-binding protein (LBP), and fibrinogen¹⁻³, accompanied by a rise in the acute-phase cytokines interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α)^{4,5}. In addition, elevated levels of APRs are noted in rheumatoid arthritis, inflammatory bowel disease, cardiovascular diseases, and obesitas.

The nature of APRs, which include protease inhibitors, complement proteins, and proteins that can bind to endotoxin, suggest an important role in innate immunity and reduction of inflammatory reactions. In this view, expression of APRs in the respiratory system, which is continuously exposed to numerous potentially harmful agents via the air inhaled, may enhance the early phase of specific local responses to injury and infection. In line with this hypothesis, there is a growing body of evidence for extra-hepatic expression of APRs. We and others previously demonstrated that human primary type II alveolar cells as well as respiratory cell lines derived from type II pneumocytes are able to produce LBP, α_1 -acid glycoprotein (AGP) and fibrinogen⁶⁻⁸. In addition, increased expression of CRP, AGP, and fibrinogen was reported after *in vitro* stimulation of alveolar macrophages^{9,10}. *In vivo*, increased BALF levels of AGP, LBP and fibrinogen were demonstrated in patients suffering from asthma^{11,12} and ARDS¹³. In addition, CRP detected in sputum of chronic bronchitis patients was shown to have anti-bactericidal activities *in vitro*¹⁴.

Chronic airway inflammation is considered to be a hallmark of COPD. Previous studies showed profound neutrophilia and increased levels of IL-8, TNF- α and soluble TNF-receptors in sputum of COPD patients versus healthy smokers, which correlated highly with each other and the degree of airflow limitation^{15,16}. As expression of APRs in the airways may enhance the early phase of specific local responses to injury and infection in COPD, the present study aims to assess levels of AGP, LBP, and CRP in induced sputum of well-characterized COPD patients and healthy smokers¹⁶. In addition, the relation between levels of APRs in induced sputum and plasma as indicator of the relationship between local and systemic acute-phase response was investigated.

MATERIAL & METHODS

Subjects

Eighteen patients with smoke-related, clinically stable COPD participated in the study.

All of the COPD patients met the American Thoracic Society (ATS) criteria for the diagnosis of COPD¹⁷. Inclusion criteria for COPD patients included stable airflow limitation with an FEV₁ of less than 70% predicted with reversibility of less than 11% predicted FEV₁ or less than 200 ml after inhaled β_2 -agonist administration and a previous history of at least 20 pack-years of smoking. Thirteen patients were prescribed combination therapy (fenoterol/ ipratropiumbromide) on a regular base, two patients were on long-acting β_2 -agonists and all other patients were prescribed bronchodilating agents on demand. Regular use of inhaled steroids, a history of respiratory diseases other than COPD, and increased respiratory complaints or respiratory tract infection during 4 weeks preceding the study were considered as criteria for exclusion.

A control group of so-called healthy smokers was recruited that consisted of 17 subjects with a normal FEV₁ and no medical history of lung disease. A smoking history of at least 15 pack-years was used as criterium for inclusion. The control subjects lived in the same geographic area as the patient population. The study was approved by the medical ethics committee of the University Hospital of Maastricht. Written informed consent was obtained from all subjects.

Pulmonary function test and blood sampling

FEV₁ was measured by trained lung function technicians using a spirometer (Masterlab, Jaeger, Würzburg, Germany) before and 15 min after inhalation of β -agonist via a metered-dose inhaler. Blood samples were collected in evacuated blood collecting tubes containing ethylene-diaminetetraacetic acid (EDTA; Sherwood Medical, St. Louis, MO) before sputum induction (8.00 – 10.00 AM). Plasma samples were stored at -80°C until analyzed.

Sputum induction and processing

Sputum was induced after a procedure as reported previously¹⁵. Briefly, subjects inhaled 3% hypertonic saline, nebulized via an ultrasonic nebulizer (NEB2000, TEFA-Portanje, Woerden, The Netherlands) during three 7-min periods. Before expectoration, subjects were instructed to mouthwash thoroughly with saline solution to minimize saliva contamination. The collected sputum was pooled and kept at 4°C for not more than 2 hours prior to further processing.

The volume of the pooled sputum (without selection of sputum plugs) was recorded, and an equal volume of 0.2% dithiothreitol (DTT; Sputolysin; Calbiochem, La Jolla, CA) was added. The samples were then mixed gently by a vortex mixer and incubated for 20 min at room temperature to ensure complete homogenization. Cell-free supernatants were frozen at -80°C until subsequent analysis. The total cell count and cell viability were assessed using a standard haemocytometer (Coulter Z1, Coulter Electronics, Mijdrecht, The Netherlands) and by trypan blue exclusion, respectively. Cytospins were stained according to the May-Grünwald-Giemsa method. Differential cell counts were carried out by one observer blinded to the clinical characteristics, counting 500 nucleated cells. The numbers of squamous cells were subtracted, and the differential cell counts were expressed as corrected percentages. A sample was considered adequate if the slides contained 15% or less

squamous epithelial cells.

Analysis of acute-phase reactants and inflammatory mediators in sputum and plasma
Acute-phase reactants. LBP, AGP, and CRP were measured in sputum supernatant and plasma using specific sandwich enzyme-linked immunosorbent assay (ELISA) as reported previously. Briefly, LBP was detected using polyclonal rabbit anti-rhLBP IgG as coating and biotin-labeled polyclonal rabbit anti-rhLBP IgG for detection¹⁸. For AGP measurement, plates were coated with polyclonal rabbit anti-hAGP and detection was carried out with a biotinylated polyclonal rabbit anti-hAGP¹⁹. The CRP concentration was measured by a polyclonal ELISA for which antibodies and standard were obtained from Dako A/S (Glostrup, Denmark)¹⁹. The lower detection limit was 1 ng/ml for LBP, 100 pg/ml for AGP, and 100 pg/ml for CRP.

Inflammatory mediators. Levels of IL-8 and IL-6 were measured using specific sandwich ELISA as described previously^{20,21}. The lower detection limit was 8 pg/ml for IL-8 and 10 pg/ml for IL-6. All sputum and plasma samples were analyzed in the same run. When sputum or plasma concentrations exceeded the upper detection limit of the assay, samples were additionally diluted and analyzed in a separate run with an overlap to correct for inter-assay variation. The intra- and inter-assay coefficients of variance of the various assays were all < 10%.

DTT effect. To study the effect of DTT on ELISA determination of the mediators assessed, standard curves of appropriate recombinant proteins were incubated with 0.1% DTT (Sputolysin) under the same conditions as sputum samples during processing (20 min at room temperature). The presence of DTT resulted in less than 5% inhibition of the detection of AGP, CRP, IL-6, and IL-8 by ELISA and less than 10% inhibition in case of LBP, indicating that DTT has little or no effect on the assays used in this study (data not shown).

Microvascular leakage

Microvascular leakage was determined in each individual as follows: sputum albumin (mg/L)/serum albumin (g/L). Albumin concentration in sputum and plasma were measured by ELISA using monoclonal antibody 4D5 (HM17) as coating and HRP-labeled polyclonal rabbit anti-human albumin IgG for detection¹⁶. The lower detection limit of the ELISA was 10 ng/ml. Presence of DTT resulted in less than 5% inhibition of albumin detection by ELISA.

Statistical analysis

Results are presented as mean $\pm$ standard deviation (SD) for normally distributed variables and median (range) otherwise. Groups were compared by analysis of covariance adjusted for age, gender, or pack-years where appropriate. Non-parametric data were compared by the Mann-Whitney U test. The chi-square test was used to compare categorical variables. Correlations between parameters were evaluated using Pearson's rank correlation analysis (Statistical Package for the Social Sciences, version 10.0 for Windows, SPSS Inc., Chicago, IL). A p-value of less than 0.05 denotes the presence of a significant statistical difference.

Table 1. Characteristics of the study groups investigated*

	COPD (n=18)	Healthy Smokers (n=17)	p Value [†]
Mean age, yr	60.9 ± 8.1	54.6 ± 6.9	0.017
Gender, M/F	16/2	6/11	0.001
Mean FEV ₁ , % predicted	55.5 ± 14.3	98.7 ± 16.2	< 0.001
Pack-years	47.9 ± 28.0	32.3 ± 13.2	0.045
Current smoker	12	10	NS [‡]
Ex-smoker	6	7	NS [‡]

* Values are means ± SD; [†] Student T-test; [‡] Chi-square test. COPD = chronic obstructive pulmonary disease; M = male; F = female; FEV₁ = forced expiratory volume in 1 second; pack-year = smoking 1 pack per day for 1 year; ex-smoker = quitting smoking for at least 1 year before the start of the study; NS = not significant.

Table 2. Sputum characteristics*

	COPD (n=18)	Healthy Smokers (n=17)	p Value [†]
Total cell count, 10 ⁶ cells/ml	4.0 ± 3.3	4.0 ± 4.1	NS
Viability, %	90.3 ± 7.8	90.3 ± 10.0	NS
AM, %	17.1 ± 10.9	36.1 ± 15.9	< 0.001
PMN, %	80.5 ± 12.4	62.4 ± 16.4	0.001
Eos, %	1.8 ± 3.6	0.7 ± 1.0	NS
Lym, %	0.7 ± 1.2	0.9 ± 0.8	NS
IL-8, ng/ml	3.7 (1.4-12.2)	2.3 (0.8-5.2)	0.008
IL-6, ng/ml	0.34 (0.16-1.26)	0.35 (0.15-1.43)	NS

* Data are presented as means ± SD for cell counts and median (range) for interleukins; [†] ANOVA adjusted for age, gender, and pack-years for cell counts and Mann-Whitney U test for interleukins. COPD = chronic obstructive pulmonary disease; AM = alveolar macrophages; PMN = neutrophils; Eos = eosinophils; Lym = lymphocytes; IL = interleukin; NS = not significant.

RESULTS

Clinical characteristics

Table 1 shows the clinical characteristics of COPD patients and healthy smokers. Patients with COPD had significant airflow limitation, whereas healthy smokers had normal FEV₁ values (% predicted) according to the selection criteria.

Sputum characteristics

All subjects included in this study tolerated well the procedure of sputum induction and an adequate specimen of sputum was collected in every participant. Sputum characteristics are shown in Table 2. Analysis of induced sputum showed a significant increase in neutrophils (%) and IL-8 levels in COPD patients versus healthy smokers, which highly correlated with each other ($r=0.704$, $p=0.001$) in COPD patients, but not in healthy smokers. In addition, the degree of airflow limitation (FEV₁ % predicted) correlated strongly with both the percentage of neutrophils ($r = -0.576$, $p=0.016$) and IL-8 levels ($r = -0.836$, $p<0.001$) in sputum of COPD patients, whereas no correlations were found in healthy smokers. In contrast to IL-8, levels of

Figure 1. Levels of (A) α_1 -glycoprotein (AGP), (B) lipopolysaccharide binding protein (LBP), and (C) C-reactive protein (CRP) in induced sputum obtained from patients with chronic obstructive pulmonary disease (COPD) and healthy smokers (HS). ° symbolizes outliers. * denotes p-value < 0.05.

Figure 2. Levels of (A) α_1 -glycoprotein (AGP), (B) lipopolysaccharide binding protein (LBP), and (C) C-reactive protein (CRP) in plasma of patients with chronic obstructive pulmonary disease (COPD) and healthy smokers (HS). ° symbolizes outliers. * denotes p-value < 0.05.

IL-6 were not significantly different between the study groups, and did not display any correlation with the FEV₁.

Acute-phase reactants in sputum

AGP, LBP, and CRP were detectable in the soluble phase of induced sputum samples of all subjects. As shown in Figure 1, sputum levels of both AGP and LBP were significantly increased in COPD patients when compared to healthy smokers. In

contrast, CRP levels in sputum of COPD patients tended to be lower in COPD versus controls, but this did not reach the level of statistical significance. Furthermore, sputum levels of AGP and LBP were positively correlated with each other, but not with sputum levels of CRP in both COPD patients and healthy smokers (data not shown).

Exploring the relationship between sputum levels of APRs and the degree of airflow limitation, we found an inverse correlation between the FEV₁ values (% predicted) and sputum AGP levels in COPD patients, whereas not in healthy smokers (Table 3). In contrast, levels of LBP and CRP in sputum were not related to airflow limitation.

Table 3. Correlations between acute-phase reactants and airflow limitation in patients with COPD and healthy smokers*

	COPD (n=18)		Healthy Smokers (n=16)	
	R Value	p Value	R Value	p Value
Sputum				
FEV ₁ / AGP	-0.674	0.003	0.243	NS
FEV ₁ / LBP	-0.399	NS	0.234	NS
FEV ₁ / CRP	0.227	NS	0.096	NS
Plasma				
FEV ₁ / AGP	0.506	0.038	-0.054	NS
FEV ₁ / LBP	0.550	0.022	0.333	NS
FEV ₁ / CRP	0.300	NS	0.037	NS

* Pearson's rank correlation. COPD = chronic obstructive pulmonary disease; AGP = α_1 -glycoprotein; CRP = C-reactive protein; LBP = lipopolysaccharide binding protein; FEV₁ = forced expiratory volume in 1 second; NS = not significant.

Acute-phase reactants in plasma

Systemic acute-phase response was assessed by measuring plasma levels of AGP, LBP, and CRP. As shown in Figure 2, plasma LBP was markedly elevated in COPD patients when compared to healthy smokers. In addition, circulating levels of AGP and CRP tended to be increased in COPD versus healthy smokers, but this did not reach significance. Circulating levels of the three APRs investigated were positively correlated with each other in COPD patients, but not in healthy smokers.

Next, we investigated the relationship between the systemic acute-phase response and the degree of airflow limitation. In contrast to sputum, circulating levels of AGP and LBP showed a positive correlation with the FEV₁ values (% predicted) in the COPD group, but not in healthy smokers (Table 3).

Relationship between local and systemic compartment

To investigate the possible relationship between local and systemic acute-phase response in COPD patients, we first determined to what extent the data are affected by microvascular leakage by measuring albumin levels in sputum and plasma. Sputum-to-plasma ratios of albumin (sputum albumin [mg/L]/serum albumin [g/L]) were not significantly different between the study groups (data not shown), indicating a similar degree of microvascular leakage in COPD patients and healthy smokers. Therefore, the values of the inflammatory mediators in sputum were not corrected for microvascular leakage.

Investigating the relationship between local and systemic acute-phase response, we found no direct correlations between sputum and plasma concentrations of each APR analyzed neither in the combined study group ([AGP]: $r = 0.007$, $p=0.963$; [LBP]: $r = 0.251$, $p=0.113$; [CRP]: $r = 0.070$, $p=0.666$) nor in the separate groups. These data suggest that APRs measured in sputum are locally produced rather than originating from the systemic compartment.

DISCUSSION

In this study, we present data on acute-phase reactants in sputum and plasma of patients with mild-to-moderate COPD and healthy smokers. AGP, CRP, and LBP were present in all induced sputum samples. Sputum levels of AGP and LBP were significantly increased in COPD patients when compared to healthy smokers, and inversely correlated with the FEV₁ values in the combined study groups. On the other hand, sputum CRP was not different between the study groups. In plasma, LBP levels were markedly elevated in COPD patients versus healthy smokers, whereas AGP and CRP tended to be increased in COPD. In contrast to sputum, AGP and LBP in plasma were positively correlated to the degree of airflow limitation in COPD. Exploring the relationship between local and systemic acute-phase response, we found no direct correlations between sputum and plasma concentrations of each APR analyzed in any study group, despite the fact that APRs were strongly related with each other in the single compartments.

Previous studies investigating APRs in lung pathology demonstrated an increase in BALF levels of AGP and LBP in patients suffering from asthma or ARDS¹¹⁻¹³. Herewith in line, we demonstrate elevated sputum levels of AGP and LBP in COPD patients versus healthy smokers, which showed an inverse correlation with the degree of airflow limitation. A similar inverse correlation was reported between FEV₁ values and IL-8 and neutrophils, which are thought to be important in airway pathogenesis in COPD^{15,16}. As the nature of APRs suggest that these proteins play a protective role in host defense²², this observation may imply that host defense increases with low FEV₁ values in COPD.

Investigating systemic inflammation in COPD patients, we previously demonstrated elevated plasma levels of CRP and LBP^{1,2}. In this study, we extended these results and showed that circulating levels of AGP and LBP were increased in mild-to-moderate COPD patients compared to healthy smokers. In contrast, no significant differences in circulating CRP levels were found. This discrepancy with previous studies may be due to differences in severity of disease, as in this study only patients with less severe COPD were included. When comparing levels of APRs in sputum and plasma, no direct correlations were found. In addition, sputum levels of AGP and LBP were inversely correlated with the degree of airflow limitation in the combined groups, whereas FEV₁ values and plasma levels of AGP and LBP were positively related. Thus, these results suggest that APRs measured in sputum may be due to local production rather than to microvascular leakage from the systemic compartment.

There is increasing evidence of extrahepatic expression of APRs, especially at the interfaces between the outer environment and the interior of the host, like lung and intestine. Intestinal epithelial cells were demonstrated to express AGP²³, LBP and serum amyloid A (SAA)²⁴. Recent studies investigating resident lung cells showed that type II pneumocytes and alveolar macrophages are able to produce different APRs like LBP, AGP, CRP, and fibrinogen⁶⁻¹⁰. Future studies are clearly required to elucidate the origin of the APRs in sputum of patients with COPD.

The question raises which role the APRs in sputum may play in the pathogenesis of COPD. As suggested by its name, LBP (LPS-binding protein) strongly enhances sensitivity of cells for LPS by facilitating binding of LPS to CD14, resulting in intracellular signaling via the TLR-4/MD-2 pathway. Binding of LBP to Gram-negative bacteria has also been reported²⁵, which was shown to enhance local inflammation as well as phagocytosis and clearance of bacteria *in vitro*^{26,27} and *in vivo*²⁸. Of interest, LBP also accelerates clearance of LPS by type II pneumocytes²⁹ as well as in the blood³⁰. Therefore, local production of LBP may play an important intermediary role in local host defense in COPD patients by assisting in the clearance of bacteria and enhancement of the local inflammatory response.

Similar to LBP, CRP is known to be involved in host defense. CRP-mediated protection includes activation of the classical complement cascade and binding to different pathogens including bacteria, fungi, and yeast. The interaction of cell-or particle-attached CRP with phagocytic cells – macrophages and neutrophils – results to increased phagocytosis. In addition, interaction with CRP was demonstrated to activate monocytes/macrophages to secrete reactive oxygen species³¹ and pro-inflammatory cytokines³², whereas CRP appears to down-regulate activation of neutrophils³³. Furthermore, CRP was found to block the attachment of *S.pneumoniae* and *H.influenzae* to human epithelial cells *in vitro*³⁴. Although local CRP levels were not increased in COPD, presence of CRP in the airways may significantly protect against invading pathogens in regular smokers in general. In this respect, CRP in sputum of chronic bronchitis patients was shown to have anti-bactericidal activities *in vitro*¹⁴.

AGP is a highly negatively charged glycoprotein (reviewed by Fournier³⁵) that was shown to have several immunomodulating effects, including inhibition of neutrophil infiltration and activation^{36,37}, modulation of LPS-induced cytokine secretion by monocytes/macrophages³⁸, and induction of macrophage derived IL-1 receptor antagonist (IL-1RA) release³⁹. Therefore, local expression of AGP may significantly protect against the deleterious effects of inflammation. Furthermore, AGP has the ability to bind and to carry numerous basic and neutral lipophilic drugs from endogenous and exogenous origin, like steroid hormones, which may be also be an important function in COPD patients who are prescribed inhaled steroids. Regular use of inhaled steroids was, however, an exclusion criterion in the present study, and can therefore not account for the increased AGP levels found in sputum of COPD patients. The immunomodulatory as well as the binding activities of AGP have been shown to be partly dependent on carbohydrate composition³⁵. In this respect, in asthma patients, fucosylation of AGP was shown to be altered and was suggested to give some additional information about the progression of the disease¹². Although beyond the scope of the present study, it would be interesting to compare the

fucosylation level of AGP in sputum and plasma in both study groups. We speculate that expression of APRs at the initial site of exposure to noxious particles may play a significant role in the host defence. In this respect, local production may result in higher concentration in the microenvironment and may shorten the time path when compared to extravasation of circulating APRs.

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REFERENCES

1. Schols AM, Buurman WA, Staal van den Brekel AJ, Dentener MA, and Wouters EF. Evidence for a relation between metabolic derangements and increased levels of inflammatory mediators in a subgroup of patients with chronic obstructive pulmonary disease. *Thorax* 1996;51:819-824.
2. Dentener MA, Creutzberg EC, Schols AM, Mantovani A, van't Veer C, Buurman WA, and Wouters EF. Systemic anti-inflammatory mediators in COPD: increase in soluble interleukin 1 receptor II during treatment of exacerbations. *Thorax* 2001;56:721-726.
3. Dahl M, Tybjaerg-Hansen A, Vestbo J, Lange P, and Nordestgaard BG. Elevated plasma fibrinogen associated with reduced pulmonary function and increased risk of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164:1008-1011.
4. Takabatake N, Nakamura H, Abe S, Inoue S, Hino T, Saito H, Yuki H, Kato S, and Tomoike H. The relationship between chronic hypoxemia and activation of the tumor necrosis factor- α system in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;161:1179-1184.
5. Eid AA, Ionescu AA, Nixon LS, Lewis-Jenkins V, Matthews SB, Griffiths TL, and Shale DJ. Inflammatory response and body composition in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;164:1414-1418.
6. Dentener MA, Vreugdenhil AC, Hoet PH, Vernooy JH, Nieman FH, Heumann D, Janssen YM, Buurman WA, and Wouters EF. Production of the acute-phase protein lipopolysaccharide-binding protein by respiratory type II epithelial cells: implications for local defense to bacterial endotoxins. *Am J Respir Cell Mol Biol* 2000;23:146-153.
7. Crestani B, Rolland C, Lardeux B, Fournier T, Bernuau D, Pous C, Vissuzaine C, Li L, and Aubier M. Inducible expression of the α 1-acid glycoprotein by rat and human type II alveolar epithelial cells. *J Immunol* 1998;160:4596-4605.
8. Guadiz G, Sporn LA, Goss RA, Lawrence SO, Marder VJ, and Simpson Haidaris PJ. Polarized secretion of fibrinogen by lung epithelial cells. *Am J Respir Cell Mol Biol* 1997;17:60-69.
9. Dong Q, and Wright JR. Expression of C-reactive protein by alveolar macrophages. *J Immunol* 1996;156:4815-4820.
10. Fournier T, Bouach N, Delafosse C, Crestani B, and Aubier M. Inducible expression and regulation of the α 1-acid glycoprotein gene by alveolar macrophages: prostaglandin E2 and cyclic AMP act as new positive stimuli. *J Immunol* 1999;163:2883-2890.
11. Dubin W, Martin TR, Swoveland P, Leturcq DJ, Moriarty AM, Tobias PS, Bleecker ER, Goldblum SE, and Hasday JD. Asthma and endotoxin: lipopolysaccharide-binding protein and soluble CD14 in bronchoalveolar compartment. *Am J Physiol* 1996;270:L736-744.
12. Van Den Heuvel MM, Poland DC, De Graaff CS, Hoefsmit EC, Postmus PE, Beelen RH, and Van Dijk W. The degree of branching of the glycans of α 1-acid glycoprotein in asthma. A correlation with lung function and inflammatory parameters. *Am J Respir Crit Care Med* 2000;161:1972-1978.

13. Martin TR, Rubenfeld GD, Ruzinski JT, Goodman RB, Steinberg KP, Leturcq DJ, Moriarty AM, Raghu G, Baughman RP, and Hudson LD. Relationship between soluble CD14, lipopolysaccharide binding protein, and the alveolar inflammatory response in patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1997;155:937-944.
14. Gould JM, and Weiser JN. Expression of C-reactive protein in the human respiratory tract. *Infect Immun* 2001;69:1747-1754.
15. Keatings VM, Collins PD, Scott DM, and Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530-534.
16. Vernooij JHJ, Küçükaycan M, Jacobs JA, Chavannes NH, Buurman WA, M.A. D, and E.F.M. W. Local and Systemic Inflammation in Patients with Chronic Obstructive Pulmonary Disease: Soluble Tumor Necrosis Factor Receptors are Increased in Sputum. *Am J Respir Crit Care Med* 2002;166:1218-1224.
17. ATS. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1995;152:S77-121.
18. Froon AH, Dentener MA, Greve JW, Ramsay G, and Buurman WA. Lipopolysaccharide toxicity-regulating proteins in bacteremia. *J Infect Dis* 1995;171:1250-1257.
19. van Dielen FM, van't Veer C, Schols AM, Soeters PB, Buurman WA, and Greve JW. Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. *Int J Obes Relat Metab Disord* 2001;25:1759-1766.
20. Bouma MG, Stad RK, van den Wildenberg FA, and Buurman WA. Differential regulatory effects of adenosine on cytokine release by activated human monocytes. *J Immunol* 1994;153:4159-4168.
21. Dentener MA, Bazil V, Von Asmuth EJ, Ceska M, and Buurman WA. Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor- alpha, IL-6 and IL-8 release by human monocytes and alveolar macrophages. *J Immunol* 1993;150:2885-2891.
22. Steel DM, and Whitehead AS. The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunol Today* 1994;15:81-88.
23. Boudreau F, Yu SJ, and Asselin C. CCAAT/enhancer binding proteins beta and delta regulate alpha1-acid glycoprotein gene expression in rat intestinal epithelial cells. *DNA Cell Biol* 1998;17:669-677.
24. Vreugdenhil AC, Dentener MA, Snoek AM, Greve JW, and Buurman WA. Lipopolysaccharide binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acute phase response. *J Immunol* 1999;163:2792-2798.
25. Lengacher S, Jongeneel CV, Le Roy D, Lee JD, Kravchenko V, Ulevitch RJ, Glauser MP, and Heumann D. Reactivity of murine and human recombinant LPS-binding protein (LBP) within LPS and gram negative bacteria. *J Inflamm* 1995;47:165-172.
26. Grunwald U, Fan X, Jack RS, Workalemahu G, Kallies A, Stelter F, and Schutt C. Monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism. *J Immunol* 1996;157:4119-4125.
27. Lazou Ahren I, Bjartell A, Egesten A, and Riesbeck K. Lipopolysaccharide-binding protein increases toll-like receptor 4-dependent activation by nontypeable Haemophilus influenzae. *J Infect Dis* 2001;184:926-930.
28. Fan MH, Klein RD, Steinstreasser L, Merry AC, Nemzek JA, Remick DG, Wang SC, and Su GL. An essential role for lipopolysaccharide-binding protein in pulmonary innate immune responses. *Shock* 2002;18:248-254.
29. Hamann L, Stamme C, Ulmer AJ, and Schumann RR. Inhibition of LPS-induced activation of alveolar macrophages by high concentrations of LPS-binding protein. *Biochem Biophys Res Commun* 2002;295:553-560.
30. Vreugdenhil AC, Snoek AM, van 't Veer C, Greve JW, and Buurman WA. LPS-binding protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction. *J Clin Invest* 2001;107:225-234.
31. Zeller JM, Landay AL, Lint TF, and Gewurz H. Enhancement of human peripheral blood monocyte respiratory burst activity by aggregated C-reactive protein. *J Leukoc Biol* 1986;40:769-783.
32. Galve-de Rochemonteix B, Wiktorowicz K, Kushner I, and Dayer JM. C-reactive protein increases production of IL-1 alpha, IL-1 beta, and TNF-alpha, and expression of mRNA by human alveolar macrophages. *J Leukoc Biol* 1993;53:439-445.
33. Volanakis JE. Human C-reactive protein: expression, structure, and function. *Mol Immunol* 2001;38:189-197.

34. Gould JM, and Weiser JN. The inhibitory effect of C-reactive protein on bacterial phosphorylcholine platelet-activating factor receptor-mediated adherence is blocked by surfactant. *J Infect Dis* 2002;186:361-371.
35. Fournier T, Medjoubi NN, and Porquet D. Alpha-1-acid glycoprotein. *Biochim Biophys Acta* 2000;1482:157-171.
36. Laine E, Couderc R, Roch-Arveiller M, Vasson MP, Giroud JP, and Raichvarg D. Modulation of human polymorphonuclear neutrophil functions by alpha 1-acid glycoprotein. *Inflammation* 1990;14:1-9.
37. Muchitsch EM, Varadi K, and Pichler L. Effects of alpha 1-acid glycoprotein on acute pancreatitis and acute lung injury in rats. *Arzneimittelforschung* 2000;50:987-994.
38. Boutten A, Dehoux M, Deschenes M, Rouzeau JD, Bories PN, and Durand G. Alpha 1-acid glycoprotein potentiates lipopolysaccharide-induced secretion of interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha by human monocytes and alveolar and peritoneal macrophages. *Eur J Immunol* 1992;22:2687-2695.
39. Bories PN, Feger J, Benbernou N, Rouzeau JD, Agneray J, and Durand G. Prevalence of tri- and tetraantennary glycans of human alpha 1-acid glycoprotein in release of macrophage inhibitor of interleukin-1 activity. *Inflammation* 1990;14:315-323.

CHAPTER 5

Leptin as local inflammatory marker in COPD

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ABSTRACT

Introduction: COPD is characterized by chronic inflammation of the lung. Leptin is a pleiotropic cytokine thought to play a role in host response to inflammation. As recent studies have shown that leptin receptors are present in the lung, this study aimed to determine if leptin is detectable in induced sputum of COPD patients and if there is a relationship between leptin and inflammatory markers in sputum. *Methods:* Sputum was induced in 14 male patients with moderate COPD (FEV₁: 58% (11) pred.). Leptin, total tumor necrosis factor (TNF)- α , soluble (s) TNF-receptors (TNF-R) 55 and 75, and C-reactive protein (CRP) were analyzed in induced sputum supernatant by ELISA. Leptin was also determined in EDTA plasma. *Results:* Leptin was detectable in induced sputum of 10 COPD patients. A significant relationship was found between sputum leptin and CRP ($r=0.94$, $p<0.000$) and total TNF- α ($r=0.69$, $p=0.006$), whereas leptin was inversely related to sTNF-R55 ($r=-0.54$, $p=0.047$) and sTNF-R75 ($r=-0.60$, $p=0.038$). Plasma leptin and sputum leptin were inversely correlated ($r=-0.64$, $p=0.013$). *Conclusion:* The present study demonstrated that leptin is detectable in induced sputum of patients with moderate COPD. The observed correlations between leptin and inflammatory markers in sputum may indicate that leptin is involved in the local inflammatory response in COPD.

INTRODUCTION

Leptin, initially discovered as a regulator of food intake and energy expenditure, is emerging as a pleiotropic cytokine. It is involved in a variety of physiological and pathological functions, including the regulation of hematopoiesis, angiogenesis, wound healing, and the immune response and inflammatory response (reviewed by Fantuzzi¹). The receptor for leptin (leptin-R) has been demonstrated to be present throughout the body, including the airways², which may suggest a specific function of leptin in the airways.

Although the pathogenesis of chronic obstructive pulmonary disease (COPD) is not clear, patients with COPD have been shown to exhibit a chronic inflammation in the airways. Elevated numbers of alveolar macrophages and CD8+ T cells have been found in the peripheral airways and lung parenchyma. In addition, an increased level of neutrophils has been demonstrated in sputum and bronchoalveolar lavage (BAL) fluid of COPD patients. Furthermore, an increase in interleukin-8 (IL-8) and tumor necrosis factor- α (TNF- α), which are assumed to act as chemo-attractants for neutrophils, has been reported in sputum of COPD patients (reviewed by Barnes³).

In order to unravel the potential role of leptin in this focus of chronic inflammation, we investigated if leptin is detectable in induced sputum of patients with COPD. If so, the second objective was to relate leptin levels to inflammatory markers in induced sputum.

MATERIAL & METHODS

The study population consisted of male patients with clinically stable moderate COPD, defined according to the criteria of the American Thoracic Society (ATS). Exclusion criteria were regular use of inhaled steroids, a history of respiratory disease other than COPD and a recent exacerbation (4 weeks prior to inclusion). Forced expiratory volume in one second (FEV₁) was calculated from the flow volume curve using a spirometer (Masterlab, Jaeger, Würzburg, Germany) applied before and 15 min. after inhalation of a β -agonist via a metered-dose inhaler. FEV₁ was expressed as the percentage of reference values. Fat-free mass (FFM; kg) was estimated using single frequency (50 kHz) bio-electric impedance analysis (Xitron Technologies, San Diego, CA, USA). The ethical review board of the University Hospital Maastricht approved the study. All subjects gave written informed consent.

Sputum was induced through inhalation of 3% hypertonic saline and processed with 0.1% dithiothreitol (DTT; Sputolysin; Calbiochem, La Jolla, CA) according to the procedure described by Keatings and coworkers⁴. Blood was collected in evacuated blood collecting tubes containing EDTA (Sherwood Medical, St. Louis, MO) in the morning before sputum induction (8.00-12.00 AM). Sputum and plasma samples were stored at -80°C until analysis.

Leptin was measured in induced sputum supernatant and plasma by sandwich

enzyme-linked immunosorbent assay (ELISA)⁵. C-reactive protein (CRP), total TNF- α (i.e. free TNF- α and TNF- α bound to soluble TNF-receptors), sTNF-R55 and sTNF-R75 were determined in induced sputum supernatant by ELISA as described⁵⁻⁷. Lower detection limits: 120 pg/ml for leptin, 100 pg/ml for CRP, 20 pg/ml for total TNF- α , 60 pg/ml for sTNF-R55, and 30 pg/ml for sTNF-R75. Presence of DTT resulted in less than 10% inhibition of the detection of inflammatory mediators (data not shown).

Results are presented as mean $\pm$ standard deviation (SD) for normally distributed variables. Plasma leptin levels were adjusted for fat mass when indicated. Correlations between parameters were calculated with Pearson's rank correlation analysis using SPSS (Statistical Package for the Social Sciences, version 10.1 for Windows, SPSS Inc., Chicago, IL, U.S.A.). Significance was assessed at a p-value of 0.05.

RESULTS

Fourteen weight-stable male patients with moderate COPD (FEV₁: 58 (11) % predicted) were included in this study. Mean smoking history of the study population was 52.8 (28.3) pack years with 4 patients having quit smoking for at least 1 year and 10 patients still smoking. Mean age was 59.9 (8.3) years and body mass index was 26.2 (3.6) kg/m². All patients tolerated the procedure of sputum induction well and produced an adequate specimen of sputum for analysis.

Leptin level in sputum was detectable in 10 of 14 patients. The mean value of leptin in sputum was 275 (135) pg/ml. In plasma, leptin was detectable in all samples, with a mean level of 6915 (3681) pg/ml. After adjustment for fat mass, which correlated positively with leptin ($r = 0.719$, $p = 0.008$), leptin level was 290 (110) pg/ml/kg. Leptin in plasma and in induced sputum were inversely correlated ($r = -0.64$, $p = 0.013$), a correlation which was no longer significant after plasma

leptin had been adjusted for fat mass. In induced sputum, leptin level was strongly correlated with the acute phase protein CRP ($r = 0.94$, $p < 0.000$) (Figure 1) and with sputum TNF- α levels ($r = 0.69$, $p = 0.006$). In contrast, it was inversely related to sTNF-R55 ($r = -0.54$, $p = 0.047$) and sTNF-R75 ($r = -0.60$, $p = 0.038$).

Figure 1. Correlation between leptin and CRP levels in induced sputum ($r = 0.94$, $p < 0.000$).

DISCUSSION

Chronic airway inflammation is considered to be a hallmark of COPD. The present study shows that leptin is detectable in induced sputum of COPD patients. Leptin was positively correlated with CRP and TNF- α , and inversely related with sTNF-receptors in sputum. Previous studies showed profound neutrophilia and increased levels of IL-8, TNF- α and soluble TNF-receptors in sputum of COPD patients versus controls, which correlated highly with each other and with the degree of airflow limitation^{4,7}. The finding that sputum leptin was positively correlated with CRP and TNF- α in these patients suggests that leptin is involved in the local inflammatory response in COPD. This hypothesis is supported by the fact that the structure of leptin and its receptor (leptin-R) resemble cytokine structures, which suggests that leptin is a member of the cytokine family.

The structure of leptin-R is homologous to gp-130, the signaling transducing subunit of IL-6 family cytokines, which are important inducers of acute-phase proteins like CRP. CRP is involved in first line of innate host defense, which includes activation of the classical complement cascade and mediating phagocytosis. Recent studies demonstrated extrahepatic expression of CRP in human respiratory epithelial cells and in stimulated human alveolar macrophages^{8,9}. In addition, CRP detected in sputum of chronic bronchitis patients was shown to have anti-bactericidal activities *in vitro*⁸. We speculate that induction of CRP at the initial site of injury may enhance the early phase of specific local responses to infection and injury. The observed correlation between sputum leptin and CRP suggest that leptin may play a functional role in the acute-phase response.

TNF- α is a pro-inflammatory cytokine with pleiotropic effects that acts via two related membrane-bound receptors. In the lung, TNF- α is mainly produced by activated macrophages in response to inflammatory stimuli and functions as a chemoattractant for inflammatory cells like neutrophils. Soluble TNF-receptors in sputum are suggested to be derived from activated neutrophils⁷, which shed TNF-receptors from the cell surface in response to inflammatory mediators. Our observation that leptin is positively correlated with TNF- α and inversely with sTNF-receptors, may seem contradictory as sTNF-receptors are generally considered to be markers of a pro-inflammatory state. However, the functional role of sTNF-receptors in COPD is still uncertain. Soluble TNF-receptors are known to compete for TNF- α with membrane-bound TNF-receptors, and may hereby play a role in regulating steady state levels of TNF- α in the lungs. In this respect, the correlations of leptin with TNF- α and sTNF-receptors suggest that shedding of TNF-receptors may function in scavenging of TNF- α , and sTNF-receptors may therefore have anti-inflammatory effects in sputum.

The inverse correlation between sputum leptin and plasma leptin found in the present study makes it unlikely that the presence of leptin in sputum is attributable to microvascular leakage from the plasma leptin pool. In this respect, leptin could be synthesized in the lung itself. Fetal rat lung fibroblasts, which are adipocyte-like cells, are reported to secrete leptin¹⁰. In addition, preliminary

observations by Bruno and co-workers suggested an increased expression of leptin in the submucosa of bronchial biopsies of patients with COPD compared to controls¹¹. Future research is needed to elucidate the origin of leptin in the lung. The presence of leptin and the high concentrations of leptin receptor found in the lung² strongly suggest a specific local function of leptin. To date, only few studies have addressed this topic. Leptin was demonstrated to stimulate cell proliferation in tracheal epithelial cells *in vitro*, suggesting that leptin may function as a growth factor helping to maintain the epithelial barrier². It could also play a role in the local immune response, as leptin receptors are found on neutrophils¹² and T-cells¹¹ and as is suggested by the results found in the present study.

Activation of neutrophil-bound leptin receptors showed an increase in production of oxidative species¹². The regulation of oxidative capacity may be a way of leptin to modulate PMN function. In addition, leptin-deficient mice are found to have impaired host defense against bacterial pneumonia with increased mortality. This was associated with reduced bacterial clearance and with defective alveolar macrophage phagocytosis *in vitro*. The addition of leptin has been found to restore this defect¹³. In this case, leptin was found to protect against the damaging effects of bacterial infection and seems to play a role in the early innate immune response.

Research in other local compartments, like the intestine and liver, which both originate from the same embryonic entodermic stem cells as the lungs, showed that leptin deficiency protects against chemically induced models of inflammatory disease like colitis and hepatotoxicity. This resistance found in ob/ob mice coincided with decreased levels of the pro-inflammatory cytokine TNF- α and decreased level of CD8+ T cells. In addition, leptin administration completely restored susceptibility to levels comparable to those of the wild type mice^{14,15}. These findings suggest a crucial role for leptin in the etiology of T cell-mediated inflammatory disease. This may be important in COPD as T cell activation was demonstrated in bronchial mucosa of subjects with chronic bronchitis. In addition, preliminary observations by Bruno and colleagues demonstrated leptin-R expression on T cells in bronchial biopsies of COPD patients¹¹. Leptin may affect lymphocytes by increasing the expression of activation markers, regulating production of cytokines by T cells, and polarizing T helper (Th) cells toward a Th1 phenotype¹².

Taken together, current data suggest that leptin may be involved in the acquired immune system in COPD. Future studies are clearly necessary to elucidate the role of leptin in airways of COPD patients.

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REFERENCES

1. Fantuzzi G, and Faggioni R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. *J Leukoc Biol* 2000;68:437-446.
2. Tsuchiya T, Shimizu H, Horie T, and Mori M. Expression of leptin receptor in lung: leptin as a growth factor. *Eur J Pharmacol* 1999;365:273-279.
3. Barnes PJ. Chronic obstructive pulmonary disease. *N Engl J Med* 2000;343:269-280.
4. Keatings VM, Collins PD, Scott DM, and Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530-534.
5. Van Dielen FM, van't Veer C, Schols AM, Soeters PB, Buurman WA, and Greve JW. Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. *Int J Obes Relat Metab Disord* 2001;25:1759-1766.
6. Leeuwenberg JF, Jeunhomme TM, and Buurman WA. Slow release of soluble TNF receptors by monocytes in vitro. *J Immunol* 1994;152:4036-4043.
7. Vernooy JH, Kucukaycan M, Jacobs JA, Chavannes NH, Buurman WA, Dentener MA, and Wouters EF. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. *Am J Respir Crit Care Med* 2002;166:1218-1224.
8. Gould JM, and Weiser JN. Expression of C-reactive protein in the human respiratory tract. *Infect Immun* 2001;69:1747-1754.
9. Dong Q, and Wright JR. Expression of C-reactive protein by alveolar macrophages. *J Immunol* 1996;156:4815-4820.
10. Torday JS, Sun H, Wang L, Torres E, Sunday ME, and Rubin LP. Leptin mediates the parathyroid hormone-related protein paracrine stimulation of fetal lung maturation. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L405-410.
11. Bruno A, Chiappara G, Chanez P, Merendino AM, Giammanco S, Bellia V, Bonsignore G, and Vignola AM. Expression of leptin and leptin receptor in bronchial biopsies of COPD and asthmatic subjects. *Am J Respir Crit Care Med* 2002;165:A559.
12. Faggioni R, Feingold KR, and Grunfeld C. Leptin regulation of the immune response and the immunodeficiency of malnutrition. *Faseb J* 2001;15:2565-2571.
13. Mancuso P, Gottschalk A, Phare SM, Peters-Golden M, Lukacs NW, and Huffnagle GB. Leptin-deficient mice exhibit impaired host defense in Gram-negative pneumonia. *J Immunol* 2002;168:4018-4024.
14. Siegmund B, Lehr HA, and Fantuzzi G. Leptin: a pivotal mediator of intestinal inflammation in mice. *Gastroenterology* 2002;122:2011-2025.
15. Faggioni R, Jones-Carson J, Reed DA, Dinarello CA, Feingold KR, Grunfeld C, and Fantuzzi G. Leptin-deficient (ob/ob) mice are protected from T cell-mediated hepatotoxicity: role of tumor necrosis factor alpha and IL-18. *Proc Natl Acad Sci U S A* 2000;97:2367-2372.

CHAPTER 6

Increased activity of matrix metalloproteinase (MMP)-8 and MMP-9 in induced sputum from patients with COPD

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ABSTRACT

Matrix metalloproteinases (MMPs) are suggested to play a critical role in the pathogenesis of chronic obstructive pulmonary disease (COPD), but quantitative data on MMP activity in airways are lacking. This study aimed to quantify collagenase (MMP-1 and MMP-8) and gelatinase (MMP-9) activity in induced sputum of 17 well-characterized mild-to-moderate COPD patients (FEV₁ 56% pred) and 17 healthy smokers (FEV₁ 99% pred). Using specific immuno-capture assays that allow determination of both active and potentially activatable (total) proteinases, we found a significant increase of both active and total activity of MMP-8 and MMP-9 in COPD patients versus healthy smokers, whereas MMP-1 activity was not increased. MMP-8 and MMP-9 (active and total) were strongly correlated, and were related to the absolute number of neutrophils in both study groups. In addition, an inverse correlation between MMP-8 and MMP-9 activity in sputum and airflow obstruction (FEV₁) was found in the combined study groups. Cellular distribution of MMP-8 and MMP-9 was assessed by immunohistochemistry on sputum cytopins. Strong positive staining for MMP-9 was found in both alveolar macrophages and neutrophils, whereas specific MMP-8 staining was exclusively observed in neutrophils. The presence of increased MMP-8 and MMP-9 activity in the airways of mild-to-moderate COPD patients supports the concept of an impaired proteinase-anti-proteinase balance in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a complex, heterogeneous respiratory disease, clinically defined by development of a progressive, largely irreversible airflow limitation and emphysematous degeneration. The pathophysiologic basis for COPD has not been elucidated, but it is generally conceived that chronic exposure to noxious particles such as those present in cigarette smoke induces a sustained and extensive inflammatory response throughout the airways, lung parenchyma, and pulmonary vasculature¹. This view is supported by recent studies demonstrating that the severity of COPD and the number of various inflammatory cells in the lung are strongly correlated²⁻⁸.

The chronic inflammatory response in COPD is associated with a proteinase-anti-proteinase imbalance that is held responsible for the destruction of lung parenchyma, but that also interferes with normal tissue function and repair through proteolysis of receptors and/or ligands^{9,10}. As yet, the actual proteolytic culprits responsible for the progression of COPD have not been identified. Traditionally, attention has been primarily focused on neutrophil elastase, but this hypothesis is hardly corroborated by clinical and experimental data.

Evidence from animal studies as well as preliminary studies in man suggests that members of the matrix metalloproteinase (MMP) family are critically involved in the pathogenesis of COPD. MMP expression is increased in emphysematous lung tissue¹¹⁻¹³ and elevated MMP levels are found in bronchoalveolar fluid of emphysematous patients^{13,14}. Furthermore, inhibition of MMP activity attenuates the emphysematous changes in an experimental model of COPD¹⁵.

MMPs comprise a large family of structurally related zinc metallo-endopeptidases with different substrate specificities, collectively able to degrade most, if not all, protein constituents of the extracellular matrix^{16,17}. The net MMP-activity represents a highly regulated process involving regulation of gene expression, activation and inhibition by specific inhibitors. The critical question, how MMP mRNA and protein expression in COPD translates into net proteinase activity, is not addressed by conventional methods assessing proteinase mRNA or protein expression. We have now used new specific assays that are able to distinguish between active and latent MMP forms, and analysed MMP collagenase (MMP-1 and MMP-8) and gelatinase (MMP-9) activity in induced sputum of well-characterized COPD patients with mild-to-moderate disease⁸ and healthy smokers matched for age and smoking history. In addition, cellular distribution of MMP-8 and MMP-9 was assessed by immunohistochemistry on cytopins of sputum cells.

MATERIAL & METHODS

Subjects

Seventeen patients with smoking-related, clinically stable COPD, which met American Thoracic Society (ATS) criteria for the diagnosis of COPD¹⁸, were included. Criteria for inclusion were stable airflow limitation with a forced

expiratory volume in 1 second (FEV₁) <70% predicted with reversibility <11% predicted FEV₁ or <200 ml following inhaled β_2 -agonist administration (Spirometer, Masterlab, Jaeger, Würzburg, Germany), and a previous history of at least 20 pack-years of smoking. Exclusion criteria were a history of respiratory disease other than COPD as well as increased respiratory complaints or respiratory tract infection during 4 weeks preceding the study. A control group of so-called healthy smokers was recruited consisting of 17 subjects with a normal FEV₁, no medical history of lung disease, and a smoking history of at least 15 pack-years. The study was approved by the medical ethics committee of the University Hospital of Maastricht. Written informed consent was obtained from all subjects.

Table 1. Characteristics of the study groups investigated*

	COPD (n=18)	Healthy Smokers (n=17)	p Value ¹
Mean age, yr	60.9 $\pm$ 8.1	54.6 $\pm$ 6.9	0.017
Gender, M/F	16/2	6/11	0.001
Mean FEV ₁ , % predicted	55.5 $\pm$ 14.3	98.7 $\pm$ 16.2	< 0.001
Pack-years	47.9 $\pm$ 28.0	32.3 $\pm$ 13.2	0.045
Current smoker	12	10	NS ²
Ex-smoker	6	7	NS ²

* Values are means $\pm$ SD; ¹ Student T-test; ² Chi-square test. COPD = chronic obstructive pulmonary disease; M = male; F = female; FEV₁ = forced expiratory volume in 1 second; pack-year = smoking 1 pack per day for 1 year; ex-smoker = quitting smoking for at least 1 year before the start of the study; NS = not significant.

Sputum induction and processing

Sputum was induced as reported by Keatings and coworkers⁶. In short, subjects inhaled 3% hypertonic saline, nebulized via an ultrasonic nebulizer (NEB2000, TEFA-Portanje, Woerden, The Netherlands) during three 7-minute periods. To minimize saliva contamination, subjects were instructed to mouthwash thoroughly with saline solution before expectoration. All subjects included in this study tolerated the procedure of sputum induction well and an adequate specimen of sputum could be collected in every participant. The collected sputum was pooled and kept at 4°C for not more than 2 hours prior to further processing.

The volume of the pooled sputum (without selection of sputum plugs) was recorded and processed with dithiothreitol (DTT; Sputolysin, Calbiochem, La Jolla, CA; final concentration 0.1%) as described⁶. The total cell count and cell viability were assessed using a standard haemocytometer (Coulter Z1, Coulter Electronics, Mijdrecht, The Netherlands) and by trypan blue exclusion, respectively. Differential cell counts (May-Grünwald Giemsa method) were carried out by one observer blinded to the clinical characteristics, counting 500 nucleated cells. The numbers of squamous cells were subtracted and the differential cell counts were expressed as corrected percentages. A sample was considered adequate if the slides contained $\leq$ 15% squamous epithelial cells.

Table 2. Total and differential cell counts in patients with COPD and healthy smokers*

	COPD (n=18)	Healthy Smokers (n=17)	p Value [†]
Total cell count, 10 ⁶ cells/ml	4.0 ± 3.3	4.0 ± 4.1	NS
Viability, %	90.3 ± 7.8	90.3 ± 10.0	NS
AM, %	17.1 ± 10.9	36.1 ± 15.9	< 0.001
PMN, %	80.5 ± 12.4	62.4 ± 16.4	0.001
Eos, %	1.8 ± 3.6	0.7 ± 1.0	NS
Lym, %	0.7 ± 1.2	0.9 ± 0.8	NS

* Data are presented as means ± SD; [†] ANOVA adjusted for age, gender, and pack-years. COPD = chronic obstructive pulmonary disease; AM = alveolar macrophages; PMN = neutrophils; Eos = eosinophils; Lym = lymphocytes; NS = not significant.

Analysis of MMP-1, MMP-8, and MMP-9 activity in sputum

Immunocapture activity assays. MMP-1, MMP-8 and MMP-9 activity assays (Amersham Biosciences, Buckinghamshire, UK) were performed according to the supplier's recommendations. These assays measure both active (mature) MMP as well as total MMP (already active plus activatable (latent) pro-MMP) activity. In brief, MMP-1, MMP-8, or MMP-9 were captured by a specific antibody immobilized on a microtiter plate. The amount of active MMP was measured directly by incubation of the captured MMP with modified pro-urokinase (UKcol), and subsequent activation of UKcol was quantified by a chromogenic substrate (S-2444). Colour development was recorded at 405 nm at different time intervals. The amount of total MMP activity was measured through activation of the latent pro-MMP by pre-incubation with 0.5 mM p-aminophenylmercuric acetate (APMA) for 2 h at 37°C before addition of modified pro-urokinase and chromogenic substrate. Activity is expressed in arbitrary units: $\Delta A_{504} \times 1000/t^2$. The lower detection limits were 0.5 units for MMP-1 and 1.5 units for MMP-8 and MMP-9.

DTT effect. In order to study the effect of DTT on both types of MMP assays, standard curves of appropriate recombinant proteins were incubated with 0.1% DTT (Sputolysin) under the same conditions as sputum samples during processing (20 min at room temperature). Presence of DTT did not affect MMP activities, indicating that DTT had no effect on the immunocapture activity assays used in this study (data not shown).

Immunohistochemistry for MMP-8, MMP-9 on sputum cytopsin

Cytospins were fixed in acetone and stored at -20°C until use. Cytospins were rehydrated in phosphate-buffered saline, pH 7.4 and endogenous peroxidase was quenched with 0.3% H₂O₂ in methanol. Cytospins were stained with rabbit anti-human MMP-8 polyclonal antibody or rabbit anti-human MMP-9 polyclonal antibody (TNO Gaubius, Leiden, The Netherlands). After washing, biotin-conjugated donkey anti-rabbit IgG antibody (Amersham Biosciences, Buckinghamshire, UK) was applied as the secondary detection antibody, followed by ABCComplex/HRP (Avidin-biotinylated horseradish peroxidase; DAKO, Glostrup, Denmark). Enzymatic reactivity was visualized with NovaRed (Vector Laboratories, Burlingame, USA). Cytospins were counterstained with hematoxylin and mounted. No significant staining was detected in slides incubated with a control antibody

instead of the primary detecting antibody, indicating the absence of significant background staining.

Statistical analysis

Results are presented as mean $\pm$ standard deviation (SD) for normally distributed variables and median (range) otherwise. Groups were compared by analysis of covariance adjusted for age, gender, or pack-years where appropriate. The Mann-Whitney U test was used to compare non-parametric data. Categorical variables were compared by the Chi-square test. Correlations between parameters were evaluated using Pearson's rank correlation analysis (Statistical Package for the Social Sciences, version 10.0.7 for Windows, SPSS Inc., Chicago, IL). A p-value below 0.05 was considered statistically significant.

RESULTS

Subject characteristics

Patients in the COPD group showed mild-to-moderate airflow obstruction, whereas healthy smokers had normal FEV₁ values (percentage predicted) according to the selection criteria (Table 1). Sputum characteristics are shown in Table 2. The degree of airflow limitation (FEV₁ percentage predicted) correlated strongly with the percentage of neutrophils ($r = -0.576$, $p=0.016$) in COPD patients, but not in healthy smokers.

Table 3. Characterization of MMP activities in sputum of patients with COPD and healthy smokers*

	COPD		Healthy Smokers		p Value ¹
	Number	Percentage	Number	Percentage	
Total MMP-1*	9/17	(53)	5/16	(31)	NS
Total MMP-8	13/17	(76)	10/17	(59)	NS
Total MMP-9	17/17	(100)	16/17	(94)	NS
Active MMP-1	2/17	(12)	2/15	(13)	NS
Active MMP-8	6/17	(35)	1/17	(6)	<0.05
Active MMP-9	13/17	(76)	7/17	(41)	<0.05

* Lower detection levels: MMP-1 0.5U; MMP-8 1.5U; MMP-9 1.5U¹ Chi-square test. COPD = chronic obstructive pulmonary disease; MMP = Matrix metalloproteinase; NS = not significant; U = arbitrary units ($\Delta A_{504} \times 1000/t^2$).

Quantitative measurement of MMP activity in sputum

Activities for MMP-9 (gelatinase B), MMP-1 and MMP-8 (collagenases) were measured by specific activity assays that are able to distinguish active and activatable enzyme (pro-enzyme) in two separate steps. MMP-9 activities were found in both COPD patients and controls. Total MMP-9 activity was significantly higher in the COPD group when compared to the control group (Figure 1, $p<0.05$) and active MMP-9 was found 76% of the COPD patients compared to 41% of the healthy smokers (Table 3, $p<0.05$).

Activities of the individual MMP collagenases were highly variable. Total MMP-1 activity was similar and low in both groups, and in a small minority of both

Figure 1. Levels of (a) total matrix metalloproteinase (MMP)-9, (b) total MMP-8, (c) total MMP-1, (d) active MMP-9, (e) active MMP-8, and (f) active MMP-1 in induced sputum obtained from patients with chronic obstructive pulmonary disease (COPD) and healthy smokers (HS). MMP activity was measured using specific immuno-capture assays. °symbolizes outliers. * $P < 0.05$ versus HS.

COPD patients and healthy smokers active MMP-1 (12% and 13% respectively) was detected. Alternatively, total MMP-8 activity was significantly higher in the COPD group (Figure 1, $p < 0.05$) and active MMP-8 was found in 35% of the COPD patients compared to 6% of the controls (Table 3, $p < 0.05$).

Correlations between sputum MMP-8 and MMP-9 activity, inflammatory cells, and the FEV₁

The active form and total activity of each MMP examined were highly correlated in both study groups. In addition, MMP-8 and MMP-9 were strongly correlated with each other, but not with MMP-1 (data not shown). A significant correlation

between both active and total MMP-8 and MMP-9 activity with the absolute number of neutrophils was observed in the sputum of COPD patients (Table 4). A similar correlation was noted in the healthy smokers, albeit that active MMP-8 was only detectable in one healthy smoker, and in contrast to the COPD patients, active MMP-9 and total MMP-9 activity were positively correlated to the absolute number of macrophages in sputum of healthy smokers (Figure 2).

Table 4. Correlations between sputum MMP activity and neutrophils in patients with COPD and healthy smokers*

	COPD (n=18)		Healthy Smokers (n=17)	
	R Value	p Value	R Value	p Value
Total MMP-8/neutrophils	0.671	0.003	0.558	0.020
Active MMP-8/neutrophils	0.646	0.005	-0.046	NS
Total MMP-9/neutrophils	0.673	0.003	0.720	0.001
Active MMP-9/neutrophils	0.698	0.002	0.707	0.002

* Pearson's rank correlation. COPD = chronic obstructive pulmonary disease; MMP = matrix metalloproteinase; NS = not significant.

In addition, the relationship between MMP activities and the degree of airflow obstruction was explored. We found that sputum activity of MMP-8 ([active MMP-8] $r = -0.356$, $p = 0.042$; [total MMP-8] $r = -0.397$, $p = 0.022$) and MMP-9 ([active MMP-9] $r = -0.337$, $p = 0.055$; [total MMP-9] $r = -0.433$, $p = 0.012$) were positively related to the degree of airflow obstruction for the complete study group. The correlations between MMP activities and airflow obstruction in the separated groups, however, failed to reach statistical significance.

Figure 2. Correlation between macrophages numbers ($10^6/\text{ml}$ sputum) and sputum levels of (a) total matrix metalloproteinase (MMP)-9 activity (COPD: $r = 0.197$, $p = 0.448$; control: $r = 0.711$, $p = 0.002$) or (b) active MMP-9 (COPD: $r = 0.373$, $p = 0.141$; control: $r = 0.519$, $p = 0.040$) in patients with chronic obstructive pulmonary disease ($\blacklozenge$) and healthy smokers ($\circ$).

MMP immunohistochemistry on cytopins

Immunohistochemistry on cytopins of sputum cells was performed to determine the cellular distribution of MMP-8 and MMP-9. As shown in Figure 3, strong positive staining for MMP-9 was observed in both alveolar macrophages and neutrophils, whereas immunoreactivity against MMP-8 was exclusively found in

neutrophils. In addition, squamous epithelial cells (Figure 3), eosinophils and lymphocytes, recognized on basis of morphology, and bronchial epithelial cells were negative for MMP-8 and MMP-9 (not shown).

Figure 3. Immunohistochemistry for (A) MMP-8 and (B) MMP-9 on cytopins of sputum cells. Note the strong positive staining for MMP-8 in neutrophils (PMN) and for MMP-9 in both macrophages (AM) and neutrophils. Staining for MMP-8 and MMP-9 was absent in squamous epithelial cells (SEC). Results are representative for both COPD patients and healthy smokers.

DISCUSSION

MMP activity represents a highly controlled process involving regulation of pro-enzyme expression and secretion, proteolytic activation of the pro-enzyme and (irreversible) inhibition by specific inhibitors. Specific ELISAs have the disadvantage that information on the active/inactive state of the proteinases is lost. Alternatively, the use of zymograms is elaborate, semi-quantitative, and does not differentiate between the active proteinase and proteinase-inhibitor complexes. In this study, we used specific immuno-capture assays that allow determination of both active as well as total (active and latent) MMPs in human body fluids and thus address the complex interaction between activation and inhibition of MMP activity. In these assays, specificity is achieved through capturing of the targeted proteinase by a specific antibody and the activity of the immobilized proteinase is subsequently assessed by proteolytic activation of a modified urokinase. The assay is insensitive to MMP-inhibitor complexes. Pro-MMP (latent) forms are measured through activation of the captured pro-enzyme through a mercury salt that allows determination of the potentially activatable proteinases.

In this paper, we studied MMP gelatinase and collagenase activity in induced sputum of patients with mild-to moderate COPD and healthy smokers. MMP-8 and MMP-9 activities were both increased in COPD patients versus healthy smokers, whereas low MMP-1 activities were present in both study groups. In

addition, net MMP-8 and MMP-9 activity was found in a number of healthy smokers and a significantly higher proportion of the COPD patients. Presence of active forms is an uncommon finding in biological fluids. Active MMPs are commonly associated with cellular activation under pathological circumstances and are therefore indicative of a proteinase-anti-proteinase imbalance. Presence of active MMP-8 and MMP-9 in both study groups may relate to smoking behaviour as cigarette smoke-conditioned culture medium has been shown to be potent cell stimulus. It is tempting to speculate that lipopolysaccharide present in cigarette smoke is responsible for the proteolytic dysbalance in smokers and to a greater extent in the COPD patients¹⁹.

These findings extend previous findings demonstrating increased MMP-9 concentrations (ELISA) in induced sputum of patients with chronic bronchitis^{20,21} and non-quantitative zymographic findings demonstrating MMP-9 in BAL fluid of COPD patients^{13,14,22}. Presence of active MMP-8 in induced sputum confirms the presupposition of Finlay and coworkers that MMP-8 constitutes the prime interstitial collagenase in emphysema²².

In contrast to MMP-8 and MMP-9, we found relative low MMP-1 activities in induced sputum samples of COPD patients representing the airway compartment²³. This observation apparently contrasts with the increased expression of MMP-1 in the alveolar compartment of patients with late stage emphysema¹². We suggest that these findings reflect spatial differences in MMP expression between the alveolar compartment and the airway compartment, but we cannot exclude that the proteinase profile changes during progression of the disease.

Our observation that activity of MMP-8 and MMP-9 correlates positively with neutrophil numbers in both study groups, suggests that MMP-8/MMP-9 activity in sputum originates from activated neutrophils. Neutrophils are thought to play an important role in the chronic inflammatory reaction present in the airways of COPD patients. In line with previous observations, a significant increase in neutrophils (percentage) was found in induced sputum of COPD patients versus healthy smokers, which highly correlated with the degree of airflow limitation^{4,6,24,25}. As sputum samples were centrifuged prior to freezing sputum to obtain cell free supernatants, the increased MMP-8 and MMP-9 activity in COPD sputum reflects enhanced MMP secretion rather than a proteinase release during freezing and thawing of the samples. This notion is supported by the finding of active forms in a higher proportion of the COPD patients. Herewith in line, complexes of human neutrophil lipocalin (HNL)/MMP-9 in BAL fluid of subjects with subclinical emphysema also suggest that MMP-9 present in BAL fluid is derived from neutrophils¹⁴.

Using immunohistochemical techniques on cytopins of sputum derived cells, we found a strong positive signal for MMP-9 in both neutrophils and alveolar macrophages, whereas other cell types like eosinophils, lymphocytes, bronchial epithelial cells, and squamous epithelial cells were negative. These results confirm the prominent role of alveolar macrophages and neutrophils in COPD as major source of MMP-9. Recent studies performing *in vitro* stimulation of alveolar macrophages isolated from BAL fluid demonstrated that these cells are a

significant source of MMP-9 in the airways of subjects with COPD^{22,26}. Alveolar macrophages from COPD patients were shown to release more MMP-9, and it is more active, than released from alveolar macrophages of healthy smokers and non-smoking individuals²⁶. Positive staining for MMP-8 was exclusively observed in neutrophils and not in other cell types present in sputum, which is consistent with the literature, as MMP-8 has been labeled as neutrophil collagenase^{16,17}.

Presence of increased MMP-8 and MMP-9 activity in the airways of mild-to-moderate COPD patients supports the concept of an impaired proteinase-anti-proteinase balance. In COPD, a MMP imbalance in the airways may account for inappropriate matrix remodeling and basement membrane disruption in both the upper and lower respiratory tract. Moreover, excess MMP activity may also contribute to the chronic inflammatory response and interfere with tissue repair through its effects on a variety of non-matrix proteins, including cytokines, chemokines, integrins, antimicrobial peptides, and other proteinases. Clearly, the precise mechanisms involving MMPs and their inhibitors in matrix remodeling and chronic inflammation in COPD, as well as their consequences in terms of abnormality of lung function require further investigations.

In summary, COPD patients had significantly higher levels of active as well as total (activatable) MMP-8 and MMP-9 in their sputum as compared to healthy smokers, whereas activity of MMP-1 was not increased. In both study groups, activity of MMP-8 and MMP-9 (active and total) showed a strong correlation with each other, and with absolute numbers of neutrophils. In addition, an inverse correlation between MMP-8 and MMP-9 activity in sputum and airflow obstruction (FEV₁) was found when values of the two study groups were combined. Immunohistochemistry demonstrated strong positive staining for MMP-9 in both alveolar macrophages and neutrophils, whereas specific staining for MMP-8 was only observed in neutrophils. The presence of increased MMP-8 and MMP-9 activity in the airways of mild-to-moderate COPD patients supports the concept of an impaired proteinase-anti-proteinase balance in COPD.

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REFERENCES

1. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, and Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001;163:1256-1276.

2. Finkelstein R, Fraser RS, Ghezzi H, and Cosio MG. Alveolar inflammation and its relation to emphysema in smokers. *Am J Respir Crit Care Med* 1995;152:1666-1672.
3. Saetta M, Di Stefano A, Turato G, Facchini FM, Corbino L, Mapp CE, Maestrelli P, Ciaccia A, and Fabbri LM. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1998;157:822-826.
4. Peleman RA, Ryttilä PH, Kips JC, Joos GF, and Pauwels RA. The cellular composition of induced sputum in chronic obstructive pulmonary disease. *Eur Respir J* 1999;13:839-843.
5. Pesci A, Balbi B, Majori M, Cacciani G, Bertacco S, Alciato P, and Donner CF. Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J* 1998;12:380-386.
6. Keatings VM, Collins PD, Scott DM, and Barnes PJ. Differences in interleukin-8 and tumor necrosis factor- α in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530-534.
7. O'Shaughnessy TC, Ansari TW, Barnes NC, and Jeffery PK. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am J Respir Crit Care Med* 1997;155:852-857.
8. Vernooy JHJ, Küçükaycan M, Jacobs JA, Chavannes NH, Buurman WA, M.A. D, and E.F.M. W. Local and Systemic Inflammation in Patients with Chronic Obstructive Pulmonary Disease: Soluble Tumor Necrosis Factor Receptors are Increased in Sputum. *Am J Respir Crit Care Med* 2002;166:1218-1224.
9. Taggart CC, Lowe GJ, Greene CM, Mulgrew AT, O'Neill SJ, Levine RL, and McElvaney NG. Cathepsin B, L, and S cleave and inactivate secretory leucoprotease inhibitor. *J Biol Chem* 2001;276:33345-33352.
10. Vandivier RW, Fadok VA, Hoffmann PR, Bratton DL, Penvari C, Brown KK, Brain JD, Accurso FJ, and Henson PM. Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. *J Clin Invest* 2002;109:661-670.
11. Ohnishi K, Takagi M, Kurokawa Y, Satomi S, and Kontinen YT. Matrix metalloproteinase-mediated extracellular matrix protein degradation in human pulmonary emphysema. *Lab Invest* 1998;78:1077-1087.
12. Imai K, Dalal SS, Chen ES, Downey R, Schulman LL, Ginsburg M, and D'Armiento J. Human collagenase (matrix metalloproteinase-1) expression in the lungs of patients with emphysema. *Am J Respir Crit Care Med* 2001;163:786-791.
13. Segura-Valdez L, Pardo A, Gaxiola M, Uhal BD, Becerril C, and Selman M. Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. *Chest* 2000;117:684-694.
14. Betsuyaku T, Nishimura M, Takeyabu K, Tanino M, Venge P, Xu S, and Kawakami Y. Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Respir Crit Care Med* 1999;159:1985-1991.
15. Zheng T, Zhu Z, Wang Z, Homer RJ, Ma B, Riese RJ, Jr., Chapman HA, Jr., Shapiro SD, and Elias JA. Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema. *J Clin Invest* 2000;106:1081-1093.
16. Handbook for proteolytic enzymes. 1998. In A.J. Barrett, N.D. Rawlings, and F. Woessner, editors. ed. Academic Press, London, UK.
17. Parks WC, and Shapiro SD. Matrix metalloproteinases in lung biology. *Respir Res* 2001;2:10-19.
18. ATS. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1995;152:S77-121.
19. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, and Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999;115:829-835.
20. Vignola AM, Riccobono L, Mirabella A, Profita M, Chanez P, Bellia V, Mautino G, D'Accardi P, Bousquet J, and Bonsignore G. Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1998;158:1945-1950.
21. Cataldo D, Munaut C, Noel A, Frankenne F, Bartsch P, Foidart JM, and Louis R. MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. *Int Arch Allergy Immunol* 2000;123:259-267.

22. Finlay GA, Russell KJ, McMahon KJ, D'Arcy E M, Masterson JB, FitzGerald MX, and O'Connor CM. Elevated levels of matrix metalloproteinases in bronchoalveolar lavage fluid of emphysematous patients. *Thorax* 1997;52:502-506.
23. Alexis NE, Hu SC, Zeman K, Alter T, and Bennett WD. Induced Sputum Derives from the Central Airways. Confirmation using a radiolabeled aerosol bolus delivery technique. *Am J Respir Crit Care Med* 2001;164:1964-1970.
24. Yamamoto C, Yoneda T, Yoshikawa M, Fu A, Tokuyama T, Tsukaguchi K, and Narita N. Airway inflammation in COPD assessed by sputum levels of interleukin-8. *Chest* 1997;112:505-510.
25. Rutgers SR, Timens W, Kaufmann HF, van der Mark TW, Koeter GH, and Postma DS. Comparison of induced sputum with bronchial wash, bronchoalveolar lavage and bronchial biopsies in COPD. *Eur Respir J* 2000;15:109-115.
26. Russell RE, Culpitt SV, DeMatos C, Donnelly L, Smith M, Wiggins J, and Barnes PJ. Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2002;26:602-609.

CHAPTER 7

Intratracheal instillation of lipopolysaccharide in mice induces apoptosis in bronchial epithelial cells: no role for TNF- α and infiltrating neutrophils

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ABSTRACT

This study investigated apoptosis in lungs after local exposure to lipopolysaccharide (LPS). Mice were instilled intratracheally with 5 μ g LPS, which corresponds to the amount acquired by smoking approximately 25 cigarettes, and killed at different time points after exposure. Our data demonstrate that local LPS exposure resulted in apoptosis in lungs from 2 h and peaked at 24 h, as detected by ligation-mediated polymerase chain reaction. Morphologic examination and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end label staining demonstrated apoptosis in bronchial epithelial cells early after intratracheal (IT) LPS challenge, whereas infiltrating neutrophils displayed positive staining at 24 and 72 h after exposure. Apoptosis in lungs clearly preceded pulmonary neutrophil infiltration, confirming that neutrophils did not contribute to pulmonary apoptosis at early time points. Further, using three experimental approaches namely, anti-tumor necrosis factor (TNF)- α treatment, IT TNF- α instillation, and TNF/lymphotoxin- α knockout mice we demonstrate that TNF- α , which was elevated in lungs at both messenger RNA and protein levels after IT LPS challenge, was no primary mediator in LPS-induced apoptosis at early time points. Thus, local LPS exposure in mice resulted in early apoptosis of bronchial epithelial cells independent of infiltrating neutrophils and TNF- α , which suggests that apoptosis of bronchial epithelium may be involved in airway injury during exposure to LPS.

INTRODUCTION

Lipopolysaccharide (LPS) is a major pathogenic factor in Gram-negative sepsis, which is characterized by shock, coagulopathy, and multiorgan dysfunction. In response to systemic LPS exposure, proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and interferon- γ are produced by the host, which have been shown to either directly or indirectly mediate many of the hemodynamic and inflammatory changes and organ damage in sepsis. Animal models of septic shock indicated that apoptosis, an active cellular process of cell death under genetic control, contributed to primary organ damage. Systemic LPS administration in mice resulted in apoptotic cell death in the endothelium of several tissues, including intestine and lung¹⁻³. *In vivo* blocking of TNF- α by anti-TNF antibody¹ or TNF-binding proteins² attenuated LPS-induced apoptotic rates, suggesting that TNF- α is the primary mediator in endothelial apoptotic cell death induced by systemically administered LPS.

The respiratory system is continuously exposed to low levels of LPS, which is ubiquitously present as a contaminant on airborne particles, including air pollution⁴, organic dusts⁵, and cigarette smoke⁶. Exposure to high LPS levels for example, agricultural workers in contact with organic dusts, or heavy smokers is known to provoke acute lung inflammation, partly initiated via the early endogenous induction of IL-1 β and TNF- α in the lung. These cytokines are thought to contribute to the pathogenesis of acute inflammation by inducing the expression of endothelial leukocyte adhesion molecules and chemokines, consequently leading to recruitment of neutrophils into alveoli. Neutrophils play a prominent role in the host defense against pathogens, but are also considered to be responsible for pulmonary injury, manifested by increased lung vascular permeability, edema, and cell death⁷. Neutrophil presence was suggested to induce apoptotic cell death in primary human bronchial epithelial cells⁸. *In vitro* studies directed at resolving tissue injury caused by LPS have indicated that LPS can directly trigger pulmonary cells to undergo apoptosis. Bingisser and coworkers showed that LPS induced apoptosis in human alveolar macrophages dose-dependently⁹. In addition, LPS was reported to cause apoptotic cell death in primary cultures of isolated bovine and sheep pulmonary artery endothelial cells, and in a bovine pulmonary artery endothelial cell line^{10,11}.

In view of the continuous exposure of the lungs to LPS, we studied whether local exposure to LPS *in vivo* results in apoptotic cell death in lungs. To this end, mice were instilled intratracheally with LPS, and apoptotic cell death in lung tissue was assessed via ligation-mediated (LM) polymerase chain reaction (PCR) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end label staining. In this study we show for the first time that local LPS exposure results in apoptotic cell death in bronchial epithelial cells at early time points after exposure. In addition, we investigated the role of infiltrating neutrophils and TNF- α in LPS-induced apoptosis and demonstrate that neither neutrophils nor TNF- α mediate apoptotic cell death in lungs at early time points.

MATERIAL & METHODS

Animals

Male Swiss mice (30 to 40 g) were obtained from Charles River Breeding Laboratories (Heidelberg, Germany). Animals were housed individually in standard laboratory cages and allowed food and water *ad libitum* throughout the experiments. The studies were carried out under a protocol approved by the Institutional Animal Care Committee of Maastricht University.

Male TNF/lymphotoxin (LT)- α double knockout (-/-) and wild type (+/+) mice were obtained from Dr. H. P. Eugster (University Hospital Zurich, Department of Internal Medicine, Zurich, Switzerland¹²). These mutant mice were bred at the Laboratory of Molecular Biology, University of Gent (Gent, Belgium) according to the Belgian and European Union guidelines for the use and care of laboratory animals. The experiments were performed in Gent under a protocol approved by the Institutional Animal Care Committee of the University of Gent.

Experimental protocol

Intratracheal (IT) instillation technique was performed according to Starcher and Williams¹³. A control experiment was performed in which bromothymol blue dissolved in 50 μ l 0.9% NaCl was instilled in mice to check distribution of solution in the lung. Macroscopic and microscopic analysis demonstrated that blue marker dye had spread throughout the whole lung. Mice ($n = 6$ per group) were anesthetized by intraperitoneal (IP) injection of 3 mg/kg xylazine (Sedamun, AUV Cuijk, The Netherlands) and 75 mg/kg ketamin (Nimatek, AUV Cuijk, The Netherlands). LPS (*Escherichia coli*, serotype O55:B5, Sigma, St. Louis, MO) dissolved in 50 μ l sterile 0.9% NaCl was instilled intratracheally via a canule, followed by 0.15 ml of air. The dose of LPS used was 5 μ g/mouse, which corresponds to the LPS dose delivered to the human lung by smoking approximately 25 cigarettes⁶. No signs of an overall toxic effect of the dose of LPS used were observed in the trachea, airways, and lungs, either in this study or in those by others^{14,15}. Sham mice were instilled intratracheally with 50 μ l LPS-free sterile 0.9% NaCl, whereas control mice received no treatment. After IT treatment, the mice were kept in an upright position for 10 min to allow the fluid to spread throughout the lungs. Mice were killed at 2, 4, 8, 24, or 72 h after instillation, and blood was collected by heart puncture. After thoracotomy, lungs were prepared for bronchoalveolar lavage (BAL) or DNA extraction, RNA extraction, myeloperoxidase (MPO) analysis, and light microscopy.

TNF- α neutralization *in vivo* was accomplished by using antimurine TNF- α monoclonal antibody (mAb) TN3, a complementarity-determining regions-grafted murine immunoglobulin (Ig) G2a (a generous gift from Celltech, Slough, UK), which was shown to have neutralizing capacities *in vivo*¹⁶. IgG class-matched control antibody (IgG2a mAb 5D7¹⁷) was used as control reagent. Administration of these reagents to mice not subjected to IT LPS instillation did not influence any of the parameters investigated (data not shown). Mice treated with anti-TNF- α mAb TN3 and control IgG2a mAb 5D7 received a single IP injection of 1 mg

antibody in 1 ml LPS-free sterile 0.9% NaCl at 2 h before IT LPS instillation. At 4 or 24 h after LPS, mice were killed and the lungs were prepared for DNA extraction and histology.

Analysis of DNA fragmentation

Genomic DNA was isolated from snap-frozen lung tissue of the right lung using a DNA purification kit (Wizard; Promega, Madison, WI). DNA concentration and purity were ascertained by electrophoresis on an ethidium bromide-stained 0.8% agarose gel followed by ultraviolet (UV) illumination and UV spectrophotometric analysis at wavelengths of 260 and 280 nm. DNA fragmentation in lung tissue was investigated with a commercially available LM-PCR assay kit (Apoalert; Clontech, Palo Alto, CA), enabling sensitive and semiquantitative measurement of the extent of apoptosis. Dephosphorylated adaptors (12- and 24-mer) were ligated to 500 ng of DNA with T4 DNA ligase for 16 h at 16°C. The 24-mer adaptor also served as primer in the LM-PCR, in which 25 ng of ligated DNA was amplified under the following conditions: hot start (72°C for 8 min) with Taq polymerase (Perkin-Elmer/Cetus, Emeryville, CA) added after 3 min, 22 cycles (94°C for 15 s, 72°C for 3 min), and postcycling (72°C for 15 min). Amplified DNA was separated by electrophoresis on a 1.2% agarose gel containing ethidium bromide and visualized by UV illumination. Intensity of the 360-base pair (bp) band was digitally analyzed (SigmaGel 1.0; SPSS, Chicago, IL) and expressed in arbitrary units.

Histology

After thoracotomy, the left lung was inflated with 10% phosphate-buffered formalin (pH 7.4) at a pressure of 20 cm H₂O through the trachea for 15 min and subsequently fixed in 10% phosphate-buffered formalin for 24 h. After paraffin embedding, 4 μ m sections were cut and stained with hematoxylin and eosin (H&E) for histologic analysis (Leica Microsystems, Cambridge, UK).

DNA nick-end labeling of tissue sections

Histologic aspects of apoptosis were studied by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL), performed according to the protocol described by Gavrieli and colleagues¹⁰ with slight modifications. Briefly, 4- μ m paraffin sections were deparaffinized and pretreated with 20 μ g/ml Proteinase K (Sigma). Endogenous peroxidase was quenched with 0.5% hydrogen peroxide (H₂O₂). Sections were incubated for 1 h at 37°C in a solution consisting of 25 mM Tris, 200 mM sodium cacodylate, 0.25 mg/ml bovine serum albumin (Sigma), 1.5 mM cobalt chloride, 40 μ M digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany), and 30 U/ml TdT (Boehringer Mannheim), pH 6.6. The labeling reaction was terminated by transferring the sections into 300 mM sodium chloride/30 mM sodium citrate for 30 min at 37°C. Incorporated digoxigenin-11-dUTP was demonstrated with peroxidase-conjugated sheep antidigoxigenin antibody (Boehringer Mannheim). The labeled antibody was visualized with diaminobenzidine (Boehringer Ingelheim, Heidelberg, Germany). Sections were

lightly counterstained with hematoxylin and mounted. Negative controls included TdT-free labeling mixture. Thymus sections from the same animal served as positive controls.

Determination of MPO

MPO was isolated from snap-frozen lung tissue of the right lung as described by Kuebler and coworkers¹⁹. Enzymatic detection of MPO was performed in a 96-well plate (Greiner, Nürtingen, Germany) as previously described²⁰. Briefly, assay mixtures consisted of 40 μ l 0.75 mM H_2O_2 in 80 mM phosphate-buffered saline (PBS) (pH 5.4) and 40 μ l sample diluted in 50 mM PBS (pH 6.0) and 0.5% hexa-1,6-bis-decyltrimethylammonium bromide (Sigma). The reaction was initiated by adding 20 μ l of 8 mM 3,3',5,5'-tetramethylbenzidine (TMB) (Boehringer Mannheim) in dimethyl sulfoxide (Sigma) and stopped after 15 min by adding 100 μ l/well 1 M H_2SO_4 . Subsequently, optical density was determined at 450 nm. All samples were assayed in triplicate. MPO activity was calculated per milligram of lung tissue and corrected for wet/dry ratios. A titration curve of horseradish peroxidase was used for the calculation of MPO activity, which is expressed in arbitrary units.

Reverse transcription/PCR

Total RNA was isolated from snap-frozen lung tissue of the right lung using a commercially available kit (SV Total RNA Isolation System; Promega). Total RNA concentration and purity were ascertained by electrophoresis on an ethidium bromide-stained 0.8% agarose gel followed by UV illumination and UV spectrophotometric analysis at wavelengths of 260 and 280 nm. The quantity of 5 μ g of total RNA was reverse transcribed in a 20- μ l volume using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (RT) (Life Technologies, Paisley, UK) according to the supplier's recommendations. PCR for murine TNF- α and β -actin was performed in a 25- μ l reaction volume containing 100 μ M of each deoxynucleotide triphosphate, 200 nM sequence-specific primers, and 0.5 U Taq DNA polymerase (Perkin-Elmer/Cetus) during 35 cycles under the following conditions: 95°C for 30 s, 60°C for 45 s, and 72°C for 30 s. PCR primers used in RT-PCR for TNF- α (307 bp) and β -actin (348 bp) were designed as previously described²⁰. Amplified PCR products were analyzed on a 1.2% agarose gel containing ethidium bromide and visualized by UV illumination. A mock PCR (without complementary DNA (cDNA)) was included to exclude contamination.

BAL

The trachea was exposed with a midline incision and cannulated with a steel catheter. The lungs were lavaged six times with sterile 0.9% NaCl at a volume of 1.5 ml/wash. The average fluid recovery was greater than 90%. The BAL fluid (BALF) was centrifuged at 1,500 rpm for 10 min at 4°C and the supernatants were stored at -20°C until analysis.

Enzyme-linked immunosorbent assay for murine TNF- α

TNF- α concentrations in BALF and plasma were determined using a specific enzyme-linked immunosorbent assay (ELISA) as described by Dentener and colleagues²¹. Briefly, 96-well immuno maxisorp plates (Nunc, Roskilde, Denmark) were coated with 5 μ g/ml hamster antimurine TNF- α mAb (TN3; kindly provided by Celltech). Murine recombinant (r) TNF- α (kindly provided by Genentech, San Francisco, CA) was used for standard titration curves. Polyclonal rabbit antimurine TNF- α (Genzyme, Cambridge, MA) was followed by peroxidase-conjugated goat antirabbit IgG (Jackson, West Grove, PA) and TMB was used as substrate. The ELISA had a lower detection limit of 50 pg/ml.

Statistical analysis

Data are expressed as means $\pm$ standard error of the mean (SEM). Statistical analysis was performed by means of Mann-Whitney U test and probability values below 0.05 were considered statistically significant.

RESULTS

Induction of apoptosis in lungs after intratracheal instillation of LPS

In the present study, apoptotic cell death after IT LPS instillation was determined by ligation-mediated PCR on DNA isolated from lung tissue. As shown in Figure 1, local LPS administration resulted in induction of DNA fragmentation ladders of approximately 180-bp multimers, which are considered to be characteristic of apoptotic cell death. DNA laddering was already evident 2 h after LPS treatment and peaked at 24 h (intensity: 360-bp band 2.7 U [2 h] versus 24.7 U [24 h]). At 72 h after exposure, DNA laddering was strongly diminished. Little or no DNA laddering was detected in lung tissue from saline-treated and control mice. These results indicate that IT LPS instillation induced apoptosis in the lung, which was already evident at early time points.

Figure 1. Induction of DNA laddering in lung tissue by IT LPS instillation. Mice received 5 μ g of LPS IT and were sacrificed at 2, 4, 8, 24 and 72 h postexposure. Control mice (C) and saline-treated mice (S) were killed after 24 h. DNA isolated from snap-frozen lung samples was amplified by LM-PCR and separated by electrophoresis on a 1.2% agarose gel containing ethidium bromide. Intensity of the 360-bp band was digitally analysed to quantify apoptosis (expressed in arbitrary units). M, molecular weight markers (range: 100–2000 bp). One representative experiment from a series of three is shown.

Figure 2. Neutrophil infiltration in lung tissue after IT LPS instillation. Lung tissue of LPS-treated and saline-treated mice was fixed in 10% formalin, processed according to standard procedure and stained with H&E. A: Saline-treated, 24 h. B: LPS-treated, 4 h. C: LPS-treated, 24 h. Bar = 60 μ m. Note the accumulation of neutrophils in the alveolar spaces at 24 h postexposure, which is absent at 4 h after LPS instillation and in saline-treated controls.

Involvement of neutrophils in LPS-induced early apoptosis in lung tissue

Several studies have reported that infiltrating neutrophils, recruited to the lungs after IT LPS instillation, are eliminated by apoptosis during the resolution of acute pulmonary inflammation^{22,23}. Further, a recent paper suggested that neutrophil presence could induce apoptotic cell death in human bronchial epithelial cells⁸. Therefore, we examined the role of neutrophils in the observed LPS-induced early apoptosis in the lung by comparing the kinetics of pulmonary apoptosis and pulmonary neutrophil influx after IT LPS instillation. Histologic assessment revealed that local LPS challenge resulted in a time-dependent neutrophil accumulation (Figure 2). Neutrophils were absent at 2 and 4 h after exposure, but presence of neutrophils in the alveolar spaces was evident from 8 h after LPS treatment and peaked at 24 h. At 72 h after exposure, neutrophil accumulation was diminished. In saline-treated and control mice, neither changes in lung histology nor a significant neutrophil influx were observed.

Next, MPO activity in lung homogenates was measured to quantify the relative neutrophil accumulation in the lung. In line with our histologic assessment of neutrophil infiltration, MPO activity was not detected in lung homogenates from LPS-treated mice at 2 and 4 h after exposure, but MPO activity increased to 17, 44, and 26 units at 8, 24, and 72 h after LPS instillation, respectively (Figure 3).

Figure 3. Quantification of pulmonary neutrophil infiltration after IT LPS instillation. Mice received 5 μ g of LPS intratracheally and were killed 2, 4, 8, 24 and 72 h after instillation. Saline-treated mice were killed 24 h after instillation. MPO activity was measured in lung homogenates to quantify the amount of infiltrated neutrophils. Data are expressed in arbitrary units (mean $\pm$ SEM). * $P < 0.05$ versus saline.

MPO activity was not demonstrated in lung tissue of saline-treated or control mice. Comparison of the kinetics of apoptosis (Figure 1) and neutrophil influx (Figure 3) in the lung revealed that apoptosis was evident from 2 h after instillation, whereas neutrophil infiltration did not occur until 8 h after LPS challenge, demonstrating that apoptosis clearly preceded the influx of neutrophils. These data show that neutrophils did not contribute to apoptotic cell death at early time points.

Figure 4. Bronchial epithelial cells undergo early apoptosis in response to local LPS administration. Lung tissue of LPS-treated and saline-treated mice was fixed in 10% formalin, processed according to standard procedure and stained by the TUNEL method. (A) Saline-treated, 4 h. (B) LPS-treated, 4 h. (C) LPS-treated, 24 h. Bar = 60 μ m. Note the brown nuclear staining in bronchial epithelial cells (arrows) at 4 and 24 h after LPS exposure, which is absent in other pulmonary cell types. Infiltrated neutrophils displayed positive staining at 24 h postexposure.

Apoptotic cell death in bronchial epithelial cells after IT LPS instillation

Next, we performed TUNEL on lung sections to determine which pulmonary cells undergo apoptosis after IT LPS treatment. As shown in Figure 4, TUNEL demonstrated intense brown nuclear staining in part of the bronchial epithelial cells from 4 h after LPS exposure, indicating apoptotic cell death. Morphologic examination demonstrated characteristic condensed chromatin in the nuclei of positive-stained bronchial epithelial cells, confirming their apoptotic state. In contrast, other pulmonary cells, e.g., alveolar type II and I cells, smooth-muscle cells, endothelial cells, and alveolar macrophages, did not display positive staining at any time point after local LPS challenge. From 24 h after exposure onward, mainly infiltrated neutrophils displayed positive staining and only few bronchial epithelial cells did so, whereas at 72 h after exposure positive nuclear staining was demonstrated only in infiltrated neutrophils. These results demonstrated early apoptosis in bronchial epithelial cells after IT LPS exposure followed by apoptosis in infiltrated neutrophils at later time points.

LPS-induced early apoptosis in lung tissue is not mediated by TNF- α

Because the proinflammatory cytokine TNF- α is suggested to be the primary mediator in endothelial apoptotic cell death after systemic administration of LPS, we investigated the role of TNF- α in the observed early apoptosis after IT LPS challenge. To confirm the presence of TNF- α in lungs after local LPS administration, we examined TNF- α protein production by specific ELISA. As shown in Table 1, IT LPS instillation resulted in elevated levels of TNF- α protein in the BALF, peaking at 4 h. TNF- α protein was not detectable in plasma, indicating

local production of TNF- α in the lung. To further establish that TNF- α was lung-derived, expression of messenger RNA (mRNA) for TNF- α was assessed by RT-PCR. Figure 5 shows enhanced pulmonary TNF- α mRNA expression at 2 h after LPS challenge, as compared with saline-treated mice.

Table 1. TNF- α levels in BALF and plasma after IT LPS challenge*

	Saline	LPS		
		4 h	24 h	72 h
BALF	< 50 [†]	650 $\pm$ 266 [†]	126 $\pm$ 72 [†]	< 50
Plasma	< 50	< 50	< 50	< 50

* Mice (n=6) received 5 μ g of IT LPS and were killed 4, 24, and 72 h after instillation. Control mice received IT saline and were killed 24 h after instillation. TNF- α was measured in BALF and plasma by specific ELISA for murine TNF- α .

[†] TNF- α is expressed in pg/ml. Data are expressed as means $\pm$ SEM.

[‡] P<0.05 versus saline.

Figure 5. Transcription of TNF- α and β -actin in murine lung tissue as determined by RT-PCR. Mice received 5 μ g of LPS or saline (S) IT and were sacrificed 2 h after instillation. Total RNA was isolated from lung tissue and analysed for TNF- α and β -actin mRNA expression using RT-PCR. Equal intensity of β -actin mRNA bands confirmed equal loading of cDNA in RT-PCR. One representative experiment from a series of three is shown.

Next, mice were instilled intratracheally with a high dose of murine rTNF- α (5 μ g) to investigate the properties of TNF- α to induce apoptosis in lungs. Because LPS-induced TNF- α production was evident from 2 h after instillation, mice were killed at 2 h after exposure, and the extent of DNA laddering in the lung was determined by LM-PCR. Figure 6A shows that local TNF- α instillation did not induce DNA laddering (intensity: 360-bp band 0.2 U [TNF- α] versus 7.5 U [LPS]). To confirm these results, TNF/LT- α double-knockout mice (-/-) and wild-type (+/+) control mice were used. Mice received LPS or saline intratracheally, and were killed at 4 h after instillation. As shown in Figure 6B, basic levels of DNA fragmentation ladders differed between TNF/LT- α -/- and +/+ mice (intensity: 360-bp band 1.0 U [S -/-] and 0.1 U [S +/+]). In spite of this difference, local LPS exposure resulted in increased DNA laddering in both TNF/LT- α -/- and +/+ mice (intensity: 360-bp band 5.7 U [LPS -/-] and 2.1 U [LPS +/+]) compared with saline-treated controls, thereby demonstrating that in the absence of TNF- α , DNA laddering was induced by IT LPS instillation. Together, these data clearly demonstrate that TNF- α is not the primary mediator in LPS-induced early apoptotic cell death in the lung.

Figure 6. TNF- α is not involved in LPS-induced early apoptosis in lung tissue. (A) TNF- α neutralisation or IT TNF- α instillation. Swiss mice received saline (lane 1), 5 μ g of LPS IT (lane 2), or mice were pre-treatment with an anti-TNF- α antibody (TN3, 1 mg/mouse) 2 h prior to LPS instillation (lane 3). Mice were sacrificed 4 h after IT treatment. An additional set of mice received 5 μ g of murine TNF- α IT (lane 4) and was sacrificed after 2 h. DNA fragmentation in lung tissue was detected by LM-PCR, and intensity of 360-bp band was analysed (expressed in arbitrary units). M, molecular weight markers (range: 100–2000 bp). Note that anti-TNF- α administration did not reduce DNA laddering induced by IT LPS, whereas IT TNF- α instillation did not result in DNA laddering. (B) LPS-induced early apoptosis in lung tissue of TNF/LT- α knockout mice. TNF/LT- α knockout (-/-) and wildtype (+/+) mice received IT saline (S) or 5 μ g of LPS (LPS), and were sacrificed 4 h postexposure. M, molecular weight markers. Note that IT LPS exposure in both TNF/LT- α -/- and +/+ resulted in increased DNA laddering. One representative experiment from a series of three is shown.

DISCUSSION

LPS is ubiquitously present as a contaminant on airborne particles, including air pollution⁴, organic dusts⁵, and cigarette smoke⁶. Local exposure to LPS is known to be associated with pulmonary cytokine production resulting in infiltration of neutrophils and pulmonary injury. However, little is known about the direct effects of LPS in pulmonary injury, such as apoptotic cell death. In this study, apoptosis in lung tissue after local (IT) exposure to LPS of 5 μ g/mouse was investigated. This amount of LPS was considered relevant, taking into account that smoking of one cigarette delivers approximately 0.2 μ g LPS to the lung⁶ and the reported 2 to 3 log less sensitivity of mice to LPS compared with humans^{24,25}. Moreover, occupational exposure to (organic dust containing) LPS is known to deliver an even higher LPS dose (30 to 60 μ g) to the lung over an 8-h work shift²⁶. To identify apoptosis in lung tissue, we used LM-PCR on genomic DNA, a very sensitive technique that enables detection of apoptosis in small percentages of cells²⁷. Interestingly, our results show that IT LPS instillation resulted in induction of DNA fragmentation ladders in lung tissue, which was already evident 2 h after LPS treatment, thereby indicating that IT LPS instillation induces apoptosis in the lung at early time points. Histologic analysis by TUNEL using the sensitive digoxigenin (DIG)-anti-DIG labeling system, which reduces the chance of detecting secondary necrotic cells²², demonstrated nuclear staining in bronchial

epithelial cells from 4 h after exposure. Additional morphological examination demonstrated characteristic condensed chromatin in the nuclei of positive-stained bronchial epithelial cells, thereby confirming their apoptotic state in response to IT LPS instillation.

Neutrophils play a prominent role in the host defense against pathogens, but are also considered to be responsible for pulmonary injury by the release of toxic contents, such as proteinases and oxygen radicals. Disruption of bronchial epithelial cell interactions with neighbor cells or extracellular matrix is known to enhance apoptosis^{10,28}, which could be one of the mechanisms for neutrophils to induce pulmonary injury. Indeed, a recent study showed that neutrophil presence induced apoptotic cell death in bronchial epithelial cells *in vitro*⁸. Our finding that apoptosis in the LPS-challenged lung clearly preceded pulmonary neutrophil infiltration excludes the possibility that neutrophils are involved in bronchial epithelial cell apoptosis at early time points. These observations are in line with a previous study, demonstrating that LPS-induced endothelial cell apoptosis *in vivo* also occurred in absence of an inflammatory response². Interestingly, TUNEL revealed that infiltrated neutrophils displayed positive staining from 24 h after LPS exposure, thereby demonstrating that apoptotic cell death in infiltrating neutrophils contributes to increased DNA laddering seen in lungs at later time points after exposure. In line with our observations in mice, infiltrated neutrophils are reported to be eliminated from sites of acute inflammation by apoptosis in rat models of acute pulmonary inflammation, induced by ozone²² or IT LPS²³ exposure. In these rats, apoptotic neutrophils become quickly engulfed and degraded by alveolar macrophages, which may explain the quick decrease in DNA laddering in lungs observed in our murine model at 72 h after LPS exposure.

TNF- α , a proinflammatory cytokine with pleiotropic effects, is known to be rapidly produced in the lung during acute pulmonary inflammation after local LPS exposure^{29,30} as was also demonstrated at both mRNA and protein levels in this study. TNF- α is also known to induce apoptosis in various cell types both *in vitro* and *in vivo*. Murine models of endotoxic shock have provided abundant evidence that TNF- α acts as the primary mediator in LPS-induced endothelial cell apoptosis in lung^{1,3}, with ceramide as the intracellular effector molecule². In contrast with the role of TNF- α in LPS-induced endothelial cell apoptosis, our findings exclude TNF- α as a primary mediator in bronchial epithelial cell apoptosis induced by IT LPS instillation. We showed that anti-TNF- α treatment did not decrease the extent of DNA laddering in lung tissue at 4 h after exposure as compared with LPS-treated controls, whereas pulmonary neutrophil infiltration was reduced 24 h after LPS exposure, thereby confirming that biologic activity of TNF- α was inhibited. These results were confirmed by experiments performed in TNF/LT- α double-knockout mice, which demonstrated that DNA fragmentation ladders in lung tissue increased 4 h after LPS exposure in both TNF/LT- α knockout and wild-type mice. Moreover, exposure of mice to a high dose of murine rTNF- α intratracheally did not increase DNA laddering in lung tissue. Mallampalli and associates³¹ recently reported that IT TNF- α instillation in rats did not increase apoptosis in

another type of pulmonary epithelial cells (alveolar type II cells) at 4 to 24 h after exposure. These studies suggest that TNF- α is not essential for epithelial cell apoptosis in the lung.

The present observations suggest a direct role for LPS in bronchial epithelial cell apoptosis. To our knowledge, only few studies have investigated the mechanisms underlying LPS-induced apoptosis *in vitro* and *in vivo*. Haimovitz-Friedman and coworkers² demonstrated that endothelial cell apoptosis induced by systemic LPS exposure is mediated by sequentially TNF- α and ceramide, which is known to act as a second messenger in pleiotropic cellular functions, including proliferation, differentiation, and apoptosis³². A possible mechanism for LPS-mediated apoptosis in epithelial cells may involve Toll-like receptor (TLR)-4, which has been identified as transmembrane coreceptor in the LPS binding protein (LBP)/CD14-dependent LPS signaling across the cell membrane³³. Recently, upregulation of TLR-4 mRNA was demonstrated in heart and lung in response to LPS administration³⁴. The observation that LPS-induced DNA laddering in endothelial cells was inhibited by addition of an anti-CD14 antibody¹¹ supports the involvement of the LBP/CD14/TLR-4 pathway in LPS-mediated apoptotic cell death. Another mechanism by which LPS could trigger apoptosis may be via directly mimicking the second messenger function of ceramide in apoptotic cell death. Lipid A is known to have strong structural similarities with ceramide³⁵. In addition, *in vitro* studies also suggested functional similarities between LPS and ceramide. Joseph and colleagues³⁵ demonstrated that lipid A and native LPS directly stimulated ceramide-activated protein kinase (CAPK) activity, a direct intracellular target for ceramide in the process of apoptosis, in human leukemia (HL-60) cells and isolated human neutrophils without generating ceramide. This CAPK activity was markedly enhanced by LBP and required CD14. The observation that LPS can be transferred into phospholipid bilayers by CD14 and LBP³⁶ supports the idea that LPS can directly interact with ceramide-responsive enzymes in the plasma membrane. Whether CD14, TLRs, and ceramide are involved in bronchial epithelial cell apoptosis observed after IT LPS exposure needs to be further elucidated.

The respiratory system is challenged continuously by LPS via inhaled air containing airborne particles and pathogens. In this study we have demonstrated that IT exposure of a relevant dose of LPS induces a mild transient acute inflammation preceded by apoptosis in part of the bronchial epithelial cells throughout the bronchial tree. Whether this early induced apoptotic cell death contributes either to tissue damage, due to disturbance of the main barrier between the environment and the respiratory system, or to innate immunity is not yet understood. However, apoptosis is increasingly being identified as a protective response by the host to pathogens, with the suicide of individual cells enhancing the survival of the multicellular organism as a whole³⁷. We consider the demonstrated apoptosis important, because clearance of harmed bronchial epithelial cells by the process of apoptosis would prevent disruption of the epithelial layer (as necrosis would do), thereby preventing the spread of pathogens. Further research needs to be performed to elucidate the role of LPS-

mediated bronchial epithelial cell apoptosis in airway injury.

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REFERENCES

1. Böhlinger I, Leist M, Gantner F, Angermüller S, Tiegs G, and Wendel A. DNA fragmentation in mouse organs during endotoxic shock. *Am J Pathol* 1996;149:1381-1393.
2. Haimovitz Friedman A, Cordon Cardo C, Bayoumy S, Garzotto M, McLoughlin M, Gallily R, Edwards CK, 3rd, Schuchman EH, Fuks Z, and Kolesnick R. Lipopolysaccharide induces disseminated endothelial apoptosis requiring ceramide generation. *J Exp Med* 1997;186:1831-1841.
3. Fujita M, Kuwano K, Kunitake R, Hagimoto N, Miyazaki H, Kaneko Y, Kawasaki M, Maeyama T, and Hara N. Endothelial cell apoptosis in lipopolysaccharide-induced lung injury in mice. *Int Arch Allergy Immunol* 1998;117:202-208.
4. Kline JN, Cowden JD, Hunninghake GW, Schutte BC, Watt JL, Wohlford Lenane CL, Powers LS, Jones MP, and Schwartz DA. Variable airway responsiveness to inhaled lipopolysaccharide. *Am J Respir Crit Care Med* 1999;160:297-303.
5. Rylander R, Haglund P, and Lundholm M. Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom. *Am Rev Respir Dis* 1985;131:209-213.
6. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, and Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999;115:829-835.
7. Wagner JG, and Roth RA. Neutrophil migration during endotoxemia. *J Leukoc Biol* 1999;66:10-24.
8. McDonald RJ, and Usachenko J. Neutrophils injure bronchial epithelium after ozone exposure. *Inflammation* 1999;23:63-73.
9. Bingisser R, Stey C, Weller M, Groscurth P, Russi E, and Frei K. Apoptosis in human alveolar macrophages is induced by endotoxin and is modulated by cytokines. *Am J Respir Cell Mol Biol* 1996;15:64-70.
10. Hoyt DG, Mannix RJ, Rusnak JM, Pitt BR, and Lazo JS. Collagen is a survival factor against LPS-induced apoptosis in cultured sheep pulmonary artery endothelial cells. *Am J Physiol* 1995;269:L171-177.
11. Frey EA, and Finlay BB. Lipopolysaccharide induces apoptosis in a bovine endothelial cell line via a soluble CD14 dependent pathway. *Microb Pathog* 1998;24:101-109.
12. Eugster HP, Müller M, Karrer U, Car BD, Schnyder B, Eng VM, Woerly G, Le Hir M, di Padova F, Aguet M, Zinkernagel R, Bluethmann H, and Ryffel B. Multiple immune abnormalities in tumor necrosis factor and lymphotoxin- α double-deficient mice. *Int Immunol* 1996;8:23-36.
13. Starcher B, and Williams I. A method for intratracheal instillation of endotoxin into the lungs of mice. *Lab Anim* 1989;23:234-240.
14. Walley KR, McDonald TE, Higashimoto Y, and Hayashi S. Modulation of proinflammatory cytokines by nitric oxide in murine acute lung injury. *Am J Respir Crit Care Med* 1999;160:698-704.
15. Shellito JE, Kolls JK, and Summer WR. Regulation of nitric oxide release by macrophages after intratracheal lipopolysaccharide. *Am J Respir Cell Mol Biol* 1995;13:45-53.

16. Bemelmans MH, Gouma DJ, Greve JW, and Buurman WA. Effect of antitumour necrosis factor treatment on circulating tumour necrosis factor levels and mortality after surgery in jaundiced mice. *Br J Surg* 1993;80:1055-1058.
17. Dentener MA, Smit FT, Francot GJ, and Buurman WA. Characterization of two monoclonal antibodies directed against bactericidal/permeability-increasing protein. *J Infect Dis* 1994;170:1483-1489.
18. Gavrieli Y, Sherman Y, and Ben Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119:493-501.
19. Kuebler WM, Abels C, Schuerer L, and Goetz AE. Measurement of neutrophil content in brain and lung tissue by a modified myeloperoxidase assay. *Int J Microcirc Clin Exp* 1996;16:89-97.
20. Daemen MA, van de Ven MW, Heineman E, and Buurman WA. Involvement of endogenous interleukin-10 and tumor necrosis factor- α in renal ischemia-reperfusion injury. *Transplantation* 1999;67:792-800.
21. Dentener MA, Greve JW, Maessen JG, and Buurman WA. Role of tumour necrosis factor in the enhanced sensitivity of mice to endotoxin after exposure to lead. *Immunopharmacol Immunotoxicol* 1989;11:321-334.
22. Ishii Y, Hashimoto K, Nomura A, Sakamoto T, Uchida Y, Ohtsuka M, Hasegawa S, and Sagai M. Elimination of neutrophils by apoptosis during the resolution of acute pulmonary inflammation in rats. *Lung* 1998;176:89-98.
23. Cox G, Crossley J, and Xing Z. Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo. *Am J Respir Cell Mol Biol* 1995;12:232-237.
24. Urbaschek B, and Urbaschek R. The inflammatory response to endotoxins. *Bibl Anat* 1979;17:74-104.
25. Berczi I, Bertok L, and Berezna T. Comparative studies on the toxicity of *Escherichia coli* lipopolysaccharide endotoxin in various animal species. *Can J Microbiol* 1966;12:1070-1071.
26. Rask Andersen A, Malmberg P, and Lundholm M. Endotoxin levels in farming: absence of symptoms despite high exposure levels. *Br J Ind Med* 1989;46:412-416.
27. Staley K, Blaschke AJ, and Chun J. Apoptotic DNA fragmentation is detected by a semi-quantitative ligation-mediated PCR of blunt DNA ends. *Cell Death Diff* 1997;4:66-75.
28. Aoshiba K, Rennard SI, and Spurzem JR. Cell-matrix and cell-cell interactions modulate apoptosis of bronchial epithelial cells. *Am J Physiol* 1997;272:L28-37.
29. Arreto C-D, Dumarey C, Nahori M-A, and Vargaftig BB. The LPS-induced neutrophil recruitment into rat air pouches is mediated by TNF: likely macrophage origin. *Med Inflamm* 1997;6:335-343.
30. Johnston CJ, Finkelstein JN, Gelein R, and Oberdorster G. Pulmonary cytokine and chemokine mRNA levels after inhalation of lipopolysaccharide in C57BL/6 mice. *Toxicol Sci* 1998;46:300-307.
31. Mallampalli RK, Peterson EJ, Carter AB, Salome RG, Mathur SN, and Koretzky GA. TNF- α increases ceramide without inducing apoptosis in alveolar type II epithelial cells. *Am J Physiol* 1999;276:L481-490.
32. Pushkareva M, Obeid LM, and Hannun YA. Ceramide: an endogenous regulator of apoptosis and growth suppression. *Immunol Today* 1995;16:294-297.
33. Hirschfeld M, Ma Y, Weis JH, Vogel SN, and Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J Immunol* 2000;165:618-622.
34. Matsumura T, Ito A, Takii T, Hayashi H, and Onozaki K. Endotoxin and cytokine regulation of toll-like receptor (TLR) 2 and TLR4 gene expression in murine liver and hepatocytes. *J Interferon Cytokine Res* 2000;20:915-921.
35. Joseph CK, Wright SD, Bornmann WG, Randolph JT, Kumar ER, Bittman R, Liu J, and Kolesnick RN. Bacterial lipopolysaccharide has structural similarity to ceramide and stimulates ceramide-activated protein kinase in myeloid cells. *J Biol Chem* 1994;269:17606-17610.
36. Wurfel MM, and Wright SD. Lipopolysaccharide-binding protein and soluble CD14 transfer lipopolysaccharide to phospholipid bilayers: preferential interaction with particular classes of lipid. *J Immunol* 1997;158:3925-3934.
37. Williams GT. Programmed cell death: a fundamental protective response to pathogens. *Trends Microbiol* 1994;2:463-464.

CHAPTER 8

Rapid expression of acute-phase reactants in lung after local LPS exposure in mice

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ABSTRACT

The respiratory system is continuously exposed to lipopolysaccharide (LPS) due to inhalation of airborne LPS present as contaminant on cigarette smoke, air pollution, organic dusts, house dust. Expression of acute-phase reactants (APRs) at the site of LPS exposure may enhance the early innate immune response to injury and infection. The present study aims to investigate pulmonary and hepatic expression of four major murine APRs in a murine model of acute lung inflammation induced by intratracheal (IT) LPS exposure. We report that local LPS exposure resulted in increased pulmonary expression of mRNA for α_1 -antitrypsin (α_1 -AT), α_1 -acid glycoprotein (AGP), and LPS-binding protein (LBP) at 4h, which was also present at 24h and 72h after LPS exposure. Little or no expression of APRs was detected in lung tissue of control mice. Although pulmonary expression of SAP mRNA was not induced by LPS exposure, systemic levels of SAP were elevated from 24h post-exposure. In addition, plasma AGP and LBP were elevated, whereas plasma α_1 -AT remained unchanged. Systemic elevation of APRs was found to be associated with increased hepatic expression of mRNA for SAP, AGP, and LBP from 24h after local LPS exposure. Furthermore, plasma IL-6 levels were increased rapidly after local LPS exposure, suggesting that IL-6 acts as mediator between the pulmonary and hepatic system. In conclusion, local LPS exposure induces rapid expression of APRs in the lung, which proceeds systemic elevation of APRs associated with hepatic APRs expression.

INTRODUCTION

Endotoxin and its purified derivative lipopolysaccharide (LPS) are major proinflammatory constituents of the outer cell wall of Gram-negative bacteria, and ubiquitously present as contaminant on airborne particles, including air pollution¹, organic dusts^{2,3}, house dust⁴, and cigarette smoke⁵. Due to inhalation of airborne LPS as well as aspiration of bacteria from commensal flora in the oral and nasal cavities, the respiratory system is continuously exposed to LPS.

In normal subjects, inhalation of endotoxin induces dose-related clinical symptoms, such as fever and fatigue, changes in lung function, and activation of the innate immune system⁶. There is, however, a considerable inter-individual variability in the sensitivity to endotoxin by inhalation, as subjects with asthma⁷ or chronic bronchitis⁸ develop airflow obstruction at a lower endotoxin dose.

Extensive studies on LPS-induced inflammation have demonstrated that LPS activates cells via a LPS binding protein (LBP)/CD14/Toll-like receptor (TLR)-4/MD-2 dependent pathway (reviewed by Alexander and Rietschel⁹). In the lung, pulmonary LPS exposure is known to result in an acute inflammatory response, which is characterized by pulmonary expression of pro-inflammatory mediators and rapid but transient neutrophil infiltration into the interstitium, alveoli, and airways¹⁰. In line herewith, bronchoalveolar neutrophil influx as well as high concentrations of interleukin (IL)-8, IL-6, IL-1 β , and tumor necrosis factor- α (TNF- α) have been measured in bronchoalveolar lavage fluid and induced sputum after inhalation of endotoxin by healthy subjects^{6,11,12}.

In addition to local inflammation, inhalation of endotoxin in normal subjects was found to be associated with rapid blood leukocytosis and neutrophilia. Furthermore, a subsequent rise in blood concentrations of two acute-phase reactants (APRs), e.g. C-reactive protein (CRP) and LBP, was noticed at 24h-48h postexposure^{6,13,14}. APRs play an important role in innate host defense and reduction of acute inflammatory reactions. For example, LBP and serum amyloid P component (SAP, functional homologue of human CRP in mice) share the capacity to bind to LPS and bacteria^{9,15}, whereas α_1 -glycoprotein (AGP) and α_1 -antitrypsin (α_1 -AT) have strong anti-inflammatory properties¹⁶⁻¹⁸. In addition, α_1 -AT inhibits neutrophil elastase, hereby providing the lung protection from proteolytic digestion that may eventually result in loss of lung recoil and emphysema¹⁹. Hepatocytes are considered to be the primary producers of circulating APRs, and regulation of the hepatic acute-phase response is largely mediated by the acute-phase cytokines tumor necrosis factor- α (TNF- α), interleukin (IL)-1 and IL-6 acting alone, in combination, or in concert with various steroid hormones²⁰.

Recent studies demonstrated that resident lung cells are able to express APRs that may contribute to the innate immune response at the site of the initial injury or infection²¹⁻²³. In view of the continuous exposure of the lung to LPS, the current study aims to investigate the contribution of the lung to the acute-phase response as part of innate immune response. To this end, a murine model of acute lung inflammation induced by local (intratracheal, IT) LPS exposure was used, in which the inciting insult is transient and the pulmonary inflammatory response is well defined¹⁰. We

composed a panel of major murine APRs, namely AGP, LBP, SAP, and α_1 -AT, which are known to be induced after systemic LPS administration in mice²⁴⁻²⁶. The expression of these APRs was assessed in lung and liver, and APR levels in plasma were measured. In addition, plasma levels of IL-6, TNF- α , soluble (s) TNF-receptors were determined to study mediators involved in systemic acute-phase response.

MATERIAL & METHODS

Experimental protocol

Male Swiss mice (8-10 weeks) from Charles River Breeding Laboratories (Heidelberg, FRG) were housed individually in standard laboratory cages and allowed food and water *ad libitum* throughout the experiments. The studies were carried out under a protocol approved by the Institutional Animal Care Committee of the Maastricht University. Mice (n=6 per group) received a single intratracheal (IT) instillation of 5 μ g LPS (*Escherichia coli*, serotype O55:B5, Sigma, St. Louis, MO) in 50 μ l 0.9% NaCl as described in detail previously¹⁰. LPS-treated mice demonstrated a profound neutrophil influx in the alveolar spaces from 8 h after instillation of LPS, peaking at 24 h. Control mice received the same volume of LPS-free sterile 0.9% NaCl or were not treated, and neither changes in lung histology nor a significant neutrophil influx were observed in these mice. Mice were killed at 4, 8, 24 or 72 h after instillation, and lungs and liver were prepared for RNA extraction. Blood was collected by heart puncture and kept at 4°C in EDTA containing tubes for not more than 1 hour prior to further processing. Plasma samples were stored at -80°C until analyzed.

Determination of plasma AGP and α_1 -AT levels by western blot analysis

Plasma samples were diluted 1:1000 in a buffer containing 60 mM Tris HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β -mercaptoethanol. After heating for 5 min at 95°C, 40 μ l of sample was separated on a 6% polyacrylamide/SDS gel by electrophoresis. Proteins were transferred to PVDF membranes (Millipore BV, Etten-Leur, The Netherlands), and washed in wash buffer (Tris-buffered Saline (TBS)/0.1%Tween20). Subsequently, nonspecific binding sites were blocked for 1 h with TBS/0.1%Tween20 containing 1% BSA and washed. Membranes were incubated for 1 h with rabbit anti-murine AGP pAb (kindly provided by Dr. P. Heegaard, Danish Veterinary Laboratory, Copenhagen, Denmark) or rabbit anti-murine α_1 -AT pAb (kindly provided by Dr. C. Libert, University of Ghent, Ghent, Belgium) diluted 1:20,000 in incubation buffer (TBS/0.1%Tween20/0.1% BSA). After washing, membranes were incubated for 1 h with peroxidase-labeled goat anti-rabbit IgG (Jackson, West Grove, PA) diluted 1:2,000 in incubation buffer. The membranes were finally developed using enhanced chemoluminescence (Pierce, Rockford IL, USA) and exposed to X-ray films. The expected size of the band is 40-43 kDa for AGP¹⁶, and 57.7 kDa for α_1 -AT²⁷.

Table 1. RT-PCR primers*

mRNA of Interest	Sequences	Annealing Temperature	Product Length
α_1 -AT	Sense: 5'-TCC CAT GAG ATC GCT ACA AAC-3' Antisense: 5'-TGA TAA TGG TTC TTG GCC TCT-3'	54 °C	350 bp
AGP	Sense: 5'-GGG GCT GTC CTA AAC CCT-3' Antisense: 5'-CAA GTC AAA GGC AAG CAT G-3'	54 °C	390 bp
LBP	Sense: 5'-GGG TGG CTG CTG AAT CTC TT-3' Antisense: 5'-TGT TGA AGG CAT CAT CTG AGA-3'	54 °C	340 bp
SAP	Sense: 5'-CTT CAC CAG CCT TCT TTC AGA-3' Antisense: 5'-ACG GAC TGT GAC TTT TGA TTG T-3'	54 °C	280 bp
IL-6	Sense: 5'-TGG GAA ATC GTG GAA ATG AGA-3' Antisense: 5'-GAG AGC ATT GGA AAT TGG GGT-3'	56 °C	318 bp
TNF	Sense: 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3' Antisense: 5'-ACA TTC GAG GCT CCA GTG AAT TCG G-3'	63 °C	307 bp
β -actin	Sense: 5'-TAA AAC GCA GCT CAG TAA CAG TCG G-3' Antisense: 5'-TGC AAT CCT GTG GCA TCC ATG AAA C-3'	60 °C	348 bp

* Primers used for RT-PCR analysis in this study. The sequences of the primers, the annealing temperature and the size of the expected RT-PCR products are illustrated.

Determination of plasma levels of IL-6, LBP, SAP, TNF- α and sTNF-receptors by ELISA

IL-6 concentration in plasma was measured by ELISA. Immunomaxisorp plates (Nunc, Roskilde, Denmark) were coated overnight with monoclonal antibody 20F3 (HM1004) specific for murine IL-6 (kindly provided by Hycult Biotechnology, Uden, The Netherlands). Diluted plasma samples and a standard dilution series with murine IL-6 (a kind gift from Dr. J. van Snick, Ludwig Institute, Brussels, Belgium) were added to the plate. IL-6 was detected with biotin-conjugated monoclonal antibody 32C11 (HM1005; Hycult Biotechnology) followed by Streptavidine-HRP (Zymed Laboratories Inc., San Francisco, CA) and 3',5,5'-tetramethylbenzidine (Kirkegaard & Perry Lab, Gaithersburg, MD) as substrate. The lower detection limit was 20 pg/ml.

Concentrations of LBP, SAP, TNF- α and soluble TNF-R55 and TNF-R75 were determined in plasma using specific enzyme linked immunosorbent assay's (ELISA) as described previously²⁸⁻³¹. The intra-assay variation was less than 10%. Lower detection limits: LBP: 100 pg/ml; SAP: 500 pg/ml; TNF- α : 20 pg/ml; sTNF-R75: 50 pg/ml; TNF-R55: 5 pg/ml.

Measurement of pulmonary and hepatic APR mRNA content

Total RNA was isolated from snap frozen lung tissue (right lung) and liver using a commercially available kit (SV Total RNA Isolation System, Promega, Madison, WI). Total RNA concentration and purity was ascertained by ultraviolet spectrophotometric analysis at wavelengths of 260 and 280 nm as well as electrophoresis on an ethidium bromide-stained 0.8% agarose gel followed by UV illumination. Five μ g of total RNA was subsequently reverse transcribed into cDNA. PCR for SAP, LBP, α_1 -AT, AGP, TNF- α , IL-6 and β -actin was performed in a 25 μ l reaction volume containing 100 μ M of each dNTP, 200 nM sequence-specific primers and 0.5 U Taq DNA polymerase

(Perkin Elmer/Cetus) during 23-40 cycles under the following conditions: 95°C 30", annealing temperature (AT) 45", 72°C 30". PCR primers used in RT-PCR for α_1 -AT, AGP, SAP, TNF- α , and β -actin were designed as previously described²⁹. PCR primers for IL-6 and LBP were developed for this study (Table 1). A mock PCR (without cDNA) was included to exclude contamination. cDNA samples were standardized based on the content of β -actin cDNA as housekeeping gene, which was evaluated by performance of a β -actin PCR on multiple dilutions of each cDNA sample. After separation on a 1.2% agarose gel, band proportions were estimated by measuring the intensity of ethidium bromide fluorescence with a CCD camera and Imagemaster VDS software (Pharmacia).

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed by means of Mann-Whitney U test (Statistical Package for the Social Sciences, version 10.0.7 for Windows, SPSS Inc., Chicago, IL). A p-value <0.05 denotes the presence of a significant statistical difference.

RESULTS

Intratracheal LPS exposure results in increased expression of α_1 -AT, AGP and LBP in lung

Recent studies demonstrated that respiratory epithelial cells and alveolar macrophages are capable of expressing APRs upon stimulation^{21,22,32}. In this view, we examined pulmonary mRNA expression of SAP, LBP, AGP, and α_1 -AT after local LPS exposure *in vivo* using RT-PCR. As shown in Figure 1, no constitutive expression for APRs was found in lung tissue of control mice. However, mRNA levels of LBP, AGP, and α_1 -AT were clearly demonstrated from 4h after LPS treatment onwards. Expression of LBP and α_1 -AT peaked at 24h, and then diminished at 72h postexposure, whereas AGP expression levels peaked at 24h-72h. In contrast, mRNA for SAP was not detected in lung tissues upon IT treatment with LPS.

Figure 1. Transcription of SAP, LBP, AGP, and α_1 -AT in lung as determined by RT-PCR. Total RNA was isolated from lung tissue of age-matched control mice [C], saline-treated mice [S], and LPS-challenged mice killed at 4h, 24h or 72h. Messenger RNA expression levels of SAP (280 bp), LBP (340 bp), AGP (390 bp), α_1 -AT (350 bp), and β -actin (348 bp) were analyzed using RT-PCR (n = 3 per group). Equal intensity of β -actin mRNA bands confirmed equal loading of cDNA in RT-PCR. One representative experiment from a series of three is shown.

Figure 2. Analysis of LBP, SAP, AGP, and α_1 -AT levels in plasma after IT LPS instillation. Mice received 5 μ g of LPS IT and were sacrificed 4, 8, 24 and 72 hours after instillation. Saline-treated mice were killed 24 hours after instillation. Plasma levels of (A) LBP and (B) SAP were measured by specific sandwich ELISA ($n=6$ per group). Data

are expressed in μ g/ml (mean $\pm$ SEM). Significant increases in mean plasma levels compared with control are indicated by * ($P<0.05$). Plasma levels of (C) AGP and α_1 -AT were determined by western blot analysis ($n=3$ per group). One representative experiment from a series of three is shown.

Intratracheal LPS exposure results in elevated plasma levels of SAP, LBP, and AGP

To address whether pulmonary LPS exposure had also extrapulmonary effects, plasma levels of AGP, SAP, LBP, and α_1 -AT were assessed by ELISA or western blot analysis. As shown in Figure 2A, local LPS administration induced a 2.5-fold elevation of circulating LBP levels at 24h and 72h post-exposure, whereas LBP levels were not raised in saline-treated mice. Similar to LBP, plasma levels of SAP and AGP increased significantly following 24h after LPS instillation, and even further increased at 72h (Figure 2B and 2C). In contrast, we did not observe any apparent raise in plasma α_1 -AT levels after local LPS exposure (Figure 2C). Taken together, these data clearly demonstrate that LPS exposure in the pulmonary compartment results in a systemic acute-phase response, characterized by increased plasma levels of SAP, LBP, and AGP.

Hepatic expression of major APR genes after IT LPS exposure

The liver is considered to be the major organ in the systemic acute-phase response. To elucidate the source of the elevated levels of APRs after local LPS exposure, we examined hepatic mRNA expression of SAP, LBP, AGP, and α_1 -AT using RT-PCR and compared APR expression and kinetics in lung and liver. As shown in Figure 3, constitutive expression of all transcripts (of the expected size according to the murine cDNA sequence) was detected in the liver tissues. Hepatic mRNA levels of SAP, LBP, and AGP were clearly enhanced at 24h and 72h after pulmonary LPS treatment as compared to saline-treated or untreated controls. In addition, AGP mRNA expression levels showed a slight, but clear, increase at 4h after exposure. In contrast,

constitutive mRNA levels for α_1 -AT were not upregulated after local LPS exposure.

Comparison of APR expression in the lung (Figure 1) and the liver (Figure 3) revealed that APRs expression patterns were different between lung and liver. Constitutive expression of all APRs examined was demonstrated in liver but not in lung of control mice. Local LPS exposure resulted in increased expression of AGP and LBP in both liver and lung, whereas mRNA for SAP was only detected in the liver. In addition, upregulation of α_1 -AT expression only occurred in the lung. Moreover, we found differences in kinetics of APR expression between liver and lung. Pulmonary LBP expression was evident as soon as 4h after LPS exposure, whereas hepatic expression of LBP did not occur until 24h after LPS challenge. In view of the pattern of circulating APRs after LPS exposure in the pulmonary compartment, these results indicate that hepatic expression of APR genes contributed to the increased plasma levels of SAP, LBP, and AGP.

Figure 3. Transcription of SAP, LBP, AGP, and α_1 -AT in liver as determined by RT-PCR. Total RNA was isolated from liver tissue of age-matched control mice [C], saline-treated mice [S], and LPS-challenged mice killed at 4h, 24h or 72h. Messenger RNA expression levels of SAP (280 bp), LBP (340 bp), AGP (390 bp), α_1 -AT (350 bp), and β -actin (348 bp) were analyzed using RT-PCR (n=3 per group). Equal intensity of β -actin mRNA bands confirmed equal loading of cDNA in RT-PCR. One representative experiment from a series of three is shown.

IL-6 links pulmonary inflammatory response and hepatic APR expression

Systemic LPS administration is known to result in a hepatic acute-phase response that is dependent on both TNF- α and IL-6^{24,33}. To examine if these cytokines are also important mediators in APR production by hepatocytes in response to pulmonary LPS exposure, we first examined mRNA expression levels of TNF- α and IL-6 in the lung using RT-PCR. As shown in Figure 4, IT LPS administration results in upregulation of TNF- α and IL-6 from 4h after exposure. Additionally, we determined IL-6 and TNF- α levels in plasma. As shown in Figure 5, circulating levels of IL-6 were significantly increased at 4h after IT LPS exposure. In contrast, TNF- α was not detectable in plasma at any time points examined. Therefore, we also measured plasma levels of soluble receptors for TNF- α (sTNF-R55 and sTNF-R75), which are considered to be markers of TNF activation. As demonstrated in Figure 5B, constitutive levels of sTNF-R were found in saline-treated and untreated control animals, but IT LPS challenge did not result in a significant increase in plasma levels of sTNF-R. Taken together, these data point at IL-6 but not TNF- α as possible mediator between lung and liver in the situation of systemic acute-phase response induced by pulmonary LPS exposure.

Figure 4. Transcription of TNF- α and IL-6 in lung as determined by RT-PCR. Total RNA was isolated from lung tissue of age-matched control mice [C], saline-treated mice [S], and LPS-challenged mice killed at 4h, 24h or 72h. Messenger RNA expression levels of TNF- α (307 bp) and IL-6 (318 bp), and β -actin (348 bp) were analyzed using RT-PCR ($n=3$ per group). Equal intensity of β -actin mRNA bands confirmed equal loading of cDNA in RT-PCR. One representative experiment from a series of three is shown.

DISCUSSION

The present study demonstrates that local exposure to LPS induced rapid expression of three major APRs, namely AGP, α_1 -AT, and LBP in the lung. To our knowledge, this is the first paper to demonstrate increased α_1 -AT expression in lung tissue as well as absence of SAP expression after IT LPS exposure. To ascertain protein production, we performed broncho-alveolar lavage (BAL) and measured acute-phase protein concentrations in BAL fluid (data not shown). Although we were able to demonstrate elevated levels of LBP in BALF from 24 h post-exposure, we considered these findings unreliable as elevated levels of IgG were also measured, indicating increased vascular permeability allowing the leakage of plasma APRs into the alveolar spaces (data not shown). A second strategy to prove local protein production was immunohistochemical detection of cytoplasmic APRs in lung sections. As APRs are known to be very sticky and positive staining in the lung may therefore be due to binding of plasma APRs to endothelial cells, lungs were flushed *ex vivo* before fix-

Figure 5. Analysis of plasma levels of IL-6, TNF- α , and sTNF-receptors after IT LPS instillation. Mice received 5 μ g of LPS IT and were sacrificed 4, 8, 24 and 72 hours after instillation. Saline-treated mice were killed 24 hours after instillation. Plasma levels of (A) IL-6 and TNF- α as well as (B) sTNF-R55 and sTNF-R75 were measured by specific sandwich ELISA ($n=6$ per group). Data are expressed as mean $\pm$ SEM. Significant increases in mean plasma levels compared with control are indicated by * ($P<0.05$); ND = not done.

ation of the tissue. Unfortunately, the background staining remained dominant despite flushing, hereby making proper judgement of the cytoplasmic staining of APRs not possible (data not shown).

Our findings are in line with previous studies in rats demonstrating upregulation of mRNA for AGP and LBP in the lung after IT LPS exposure^{22,34}. In addition, recent *in vitro* studies suggested that resident lung cells are able to express APRs. We and others previously reported that stimulation of human primary type II alveolar cells as well as respiratory cell lines derived from type II pneumocytes resulted in increased levels of LBP, AGP and fibrinogen²¹⁻²³. In addition, human alveolar macrophages were reported to express genes for AGP, CRP and α_1 -AT upon stimulation^{32,35,36}. Furthermore, human microvascular endothelial cells were reported to produce AGP³⁷. We therefore speculate that resident lung cells are a possible source of APRs in the lung after local LPS exposure.

The present study demonstrates that local LPS exposure has also extrapulmonary effects. We found elevated levels of AGP, LBP, and SAP in the circulation after exposure to LPS in the pulmonary compartment. This is in line with previous studies, showing elevated plasma levels of LBP and CRP (closely related to SAP) after inhalation of endotoxin in healthy human subjects^{6,13,14}. In addition, pulmonary infection with *Pasteurella haemolytica* in calves resulted in 4-fold increase in serum LBP levels³⁸. Comparison of systemic acute-phase response induced by local and systemic LPS administration in mice reveals that local LPS exposure results in a relative mild acute-phase response, as up to hundred-fold elevated serum levels of APRs are reported after systemic LPS in mice^{24,39}. However, it is not clear if this discrepancy is caused by the route of administration, or by relative high doses of LPS used in sepsis models.

When comparing APR expression and kinetics in lung and liver to elucidate the source of the elevated circulating APRs after local LPS exposure, we found that the hepatic APR expression pattern was consistent with the APR levels found in plasma. This indicates that circulating APRs elevated upon treatment with LPS originate from hepatocytes. We speculate that IL-6 is one possible mediator between the pulmonary and hepatic system, because circulating levels of IL-6 – not TNF- α – were found to be increased after local LPS exposure despite significant upregulation of mRNA levels for both IL-6 and TNF- α in the lung. This discrepancy may be due to the time points chosen, through which we may have missed an early peak of TNF- α in plasma at 1-2 hours. On the other hand, Nelson and colleagues reported compartmentalization of TNF- α induced by either intra-alveolar or systemic LPS exposure⁴⁰. Additionally, the fact that plasma levels of both sTNF-receptors, considered to be markers of TNF activation, remained unchanged, suggests that IL-6 but not TNF- α is involved in the hepatic acute-phase response induced by pulmonary LPS exposure. However, we cannot exclude that additional mediators not investigated in this study may also be involved.

The question raises as to which physiologic role APRs produced by lung and liver may play in the innate immune response against Gram-negative bacteria. LBP and SAP are major acute-phase reactants in the mouse that share the capacity to bind to LPS^{9,15}. LBP strongly enhances sensitivity of cells for LPS by facilitating binding of LPS

to CD14, resulting in intracellular signalling via the TLR-4/MD-2 pathway. Binding of LBP to gram-negative bacteria has also been reported⁴¹, which results in phagocytosis and clearance of these microorganisms⁴². Therefore, local production of LBP may assist in the clearance of bacteria as well as enhance the local inflammatory response. In addition, LBP accelerates the transport of LPS into lipoproteins in the blood, hereby leading to neutralization of LPS leaking from the pulmonary compartment^{43,44}.

AGP is a highly negatively charged glycoprotein (reviewed by Fournier¹⁶) that was shown to inhibit neutrophil activation⁴⁵, modulate LPS-induced cytokine secretion by monocytes/macrophages⁴⁶, and induce macrophage derived IL-1 receptor antagonist (IL-1RA) release⁴⁷. In addition, *in vivo* studies demonstrated exogenous AGP to reduce leukocyte infiltration and alveolar edema in LPS-induced acute lung injury⁴⁸ and to prevent apoptosis and inflammation during renal ischemia/reperfusion injury²⁹. This suggests that local expression of AGP may significantly protect against the deleterious effects of inflammation in the alveolar space, where the integrity of the structure is essential for the maintenance of lung function. Systemically, rapid induction of serum AGP levels has been reported to protect mice from lethal Gram-negative infection⁴⁹. In this view, elevated circulating AGP may act as a negative feedback mechanism aimed at limiting the extent of the systemic inflammatory reaction.

In contrast to LBP and AGP, the physiological functions of SAP, a member of the family of pentraxins, are still not completely understood. Binding of SAP to numerous ligands has been reported, including LPS and bacteria (reviewed by De Haas⁵⁰). In addition, De Haas et al. demonstrated that a SAP-derived peptide protected against LPS-induced septic shock in mice^{15,51}. Therefore, systemic elevation of SAP induced by LPS exposure to the lung may play a role in the binding as well as clearance of host- or pathogen-derived cellular debris.

The major function of α_1 -AT is inhibition of neutrophil elastase, hereby providing the lung protection from proteolytic digestion by harmful proteases that may eventually result in loss of lung recoil and emphysema¹⁹. Furthermore, α_1 -AT is known to inhibit neutrophil superoxide production¹⁷, and induce macrophage-derived IL-1RA release¹⁸. In these respects, expression of α_1 -AT at the initial site of injury, like LBP and AGP, may have anti-inflammatory properties protecting against the deleterious effects of the acute inflammatory response.

Taken together, locally produced APRs may control the early phase of specific local responses to injury and infection before inflammation induces capillary leakage and extravasation of serum proteins. The hepatic acute-phase response may occur as a late event preventing further spreading of the LPS and local inflammation. As local production of APRs results in higher concentrations in the intra-alveolar compartment and also shortens the time path, this would be more efficient than extravasation of serum APRs. Further studies are clearly required to study the contribution of individual APRs produced in lungs to inflammation induced by exposure to bacterial endotoxin.

In summary, the current study shows that local LPS exposure in mice resulted in a rapid increase of mRNA expression for three major APRs (α_1 -AT, AGP, and LBP) in the lung. In addition, exposure to LPS in the pulmonary compartment resulted in elevated levels of AGP, LBP, and SAP in the circulation, which was consistent with the APR

expression pattern in the liver. Furthermore, we demonstrated increased plasma IL-6 levels after local LPS exposure, suggesting that IL-6 acts as mediator between the pulmonary and hepatic system. In clinical medicine, measurement of serum APRs is considered to be a valuable tool for rapid diagnosis of infection, monitoring of the disease and the patient's recovery. On the basis of the observed differences between the systemic acute-phase response and the local acute-phase response in mice, we suggest that the comparison of APRs present in bronchoalveolar lavage (BAL) fluid and serum could potentially give information on the origin of an inflammatory response due to infection being either local or systemic. Studies in patients are currently being performed to explore the application of APR pattern analysis in clinical medicine.

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REFERENCES

1. Kline JN, Cowden JD, Hunninghake GW, Schutte BC, Watt JL, Wohlford Lenane CL, Powers LS, Jones MP, and Schwartz DA. Variable airway responsiveness to inhaled lipopolysaccharide. *Am J Respir Crit Care Med* 1999;160:297-303.
2. Rylander R, Haglund P, and Lundholm M. Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom. *Am Rev Respir Dis* 1985;131:209-213.
3. Del A, Godshali MA, and Palmgren MS. Gram-negative bacterial endotoxins in grain elevator dusts. *Am Ind Hyg Assoc J* 1984;45:336-339.
4. Michel O, Kips J, Duchateau J, Vertongen F, Robert L, Collet H, Pauwels R, and Sergysels R. Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med* 1996;154:1641-1646.
5. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, and Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999;115:829-835.
6. Michel O, Nagy AM, Schroeven M, Duchateau J, Neve J, Fondu P, and Sergysels R. Dose-response relationship to inhaled endotoxin in normal subjects. *Am J Respir Crit Care Med* 1997;156:1157-1164.
7. Michel O, Duchateau J, and Sergysels R. Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. *J Appl Physiol* 1989;66:1059-1064.
8. Cavagna G, Foa V, and Vigliani EC. Effects in man and rabbits of inhalation of cotton dust or extracts and purified endotoxins. *Br J Ind Med* 1969;26:314-321.
9. Alexander C, and Rietschel ET. Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res* 2001;7:167-202.
10. Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA, and Wouters EF. Intratracheal Instillation of Lipopolysaccharide in Mice Induces Apoptosis in Bronchial Epithelial Cells. No role for tumor necrosis factor-alpha and infiltrating neutrophils. *Am J Respir Cell Mol Biol* 2001;24:569-576.
11. Clapp WD, Thorne PS, Frees KL, Zhang X, Lux CR, and Schwartz DA. The effects of inhalation of grain dust extract and endotoxin on upper and lower airways. *Chest* 1993;104:825-830.
12. Jagielo PJ, Thorne PS, Watt JL, Frees KL, Quinn TJ, and Schwartz DA. Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects. *Chest* 1996;110:263-270.

13. Michel O, Dentener M, Corazza F, Buurman W, and Rylander R. Healthy subjects express differences in clinical responses to inhaled lipopolysaccharide that are related with inflammation and with atopy. *J Allergy Clin Immunol* 2001;107:797-804.
14. Michel O, Duchateau J, Plat G, Cantinieaux B, Hotimsky A, Gerain J, and Sergysels R. Blood inflammatory response to inhaled endotoxin in normal subjects. *Clin Exp Allergy* 1995;25:73-79.
15. De Haas CJ, van der Tol ME, Van Kessel KP, Verhoef J, and Van Strijp JA. A synthetic lipopolysaccharide-binding peptide based on amino acids 27-39 of serum amyloid P component inhibits lipopolysaccharide-induced responses in human blood. *J Immunol* 1998;161:3607-3615.
16. Fournier T, Medjoubi NN, and Porquet D. Alpha-1-acid glycoprotein. *Biochim Biophys Acta* 2000;1482:157-171.
17. Bucurenci N, Blake DR, Chidwick K, and Winyard PG. Inhibition of neutrophil superoxide production by human plasma alpha 1-antitrypsin. *FEBS Lett* 1992;300:21-24.
18. Tilg H, Vannier E, Vachino G, Dinarello CA, and Mier JW. Antiinflammatory properties of hepatic acute phase proteins: preferential induction of interleukin 1 (IL-1) receptor antagonist over IL-1 beta synthesis by human peripheral blood mononuclear cells. *J Exp Med* 1993;178:1629-1636.
19. Reid PT, and Sallenave JM. Neutrophil-derived elastases and their inhibitors: potential role in the pathogenesis of lung disease. *Curr Opin Investig Drugs* 2001;2:59-67.
20. Suffredini AF, Fantuzzi G, Badolato R, Oppenheim JJ, and O'Grady NP. New insights into the biology of the acute phase response. *J Clin Immunol* 1999;19:203-214.
21. Dentener MA, Vreugdenhil AC, Hoet PH, Vernooy JH, Nieman FH, Heumann D, Janssen YM, Buurman WA, and Wouters EF. Production of the acute-phase protein lipopolysaccharide-binding protein by respiratory type II epithelial cells: implications for local defense to bacterial endotoxins. *Am J Respir Cell Mol Biol* 2000;23:146-153.
22. Crestani B, Rolland C, Lardeux B, Fournier T, Bernuau D, Pous C, Vissuzaine C, Li L, and Aubier M. Inducible expression of the alpha1-acid glycoprotein by rat and human type II alveolar epithelial cells. *J Immunol* 1998;160:4596-4605.
23. Guadiz G, Sporn LA, Goss RA, Lawrence SO, Marder VJ, and Simpson Haidaris PJ. Polarized secretion of fibrinogen by lung epithelial cells. *Am J Respir Cell Mol Biol* 1997;17:60-69.
24. Bopst M, Haas C, Car B, and Eugster HP. The combined inactivation of tumor necrosis factor and interleukin-6 prevents induction of the major acute phase proteins by endotoxin. *Eur J Immunol* 1998;28:4130-4137.
25. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, and Achong MK. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* 1998;101:311-320.
26. Schumann RR, and Zweigner J. A novel acute-phase marker: lipopolysaccharide binding protein (LBP). *Clin Chem Lab Med* 1999;37:271-274.
27. Minnich M, Kueppers F, and James H. Alpha-1-antitrypsin from mouse serum isolation and characterization. *Comp Biochem Physiol B* 1984;78:413-419.
28. Le Roy D, Glauser MP, and Heumann D. LPS binding protein (LBP) function in germfree mice. *J Endotox Res* 1997;4:183-187.
29. Daemen MA, Heemskerk VH, van't Veer C, Denecker G, Wolfs TG, Vandenabeele P, and Buurman WA. Functional protection by acute phase proteins alpha(1)-acid glycoprotein and alpha(1)-antitrypsin against ischemia/reperfusion injury by preventing apoptosis and inflammation. *Circulation* 2000;102:1420-1426.
30. Dentener MA, Greve JW, Maessen JG, and Buurman WA. Role of tumour necrosis factor in the enhanced sensitivity of mice to endotoxin after exposure to lead. *Immunopharmacol Immunotoxicol* 1989;11:321-334.
31. Bemelmans MH, Gouma DJ, and Buurman WA. LPS-induced sTNF-receptor release in vivo in a murine model. Investigation of the role of tumor necrosis factor, IL-1, leukemia inhibiting factor, and IFN-gamma. *J Immunol* 1993;151:5554-5562.
32. Fournier T, Bouach N, Delafosse C, Crestani B, and Aubier M. Inducible expression and regulation of the alpha 1-acid glycoprotein gene by alveolar macrophages: prostaglandin E2 and cyclic AMP act as new positive stimuli. *J Immunol* 1999;163:2883-2890.
33. Sharma RJ, Macallan DC, Sedgwick P, Remick DG, and Griffin GE. Kinetics of endotoxin-induced acute-phase protein gene expression and its modulation by TNF-alpha monoclonal antibody. *Am J Physiol* 1992;262:R786-793.

34. Klein RD, Su GL, Aminlari A, Alarcon WH, and Wang SC. Pulmonary LPS-binding protein (LBP) upregulation following LPS-mediated injury. *J Surg Res* 1998;78:42-47.
35. Dong Q, and Wright JR. Expression of C-reactive protein by alveolar macrophages. *J Immunol* 1996;156:4815-4820.
36. Mornex JF, Chytil-Weir A, Martinet Y, Courtney M, LeCocq JP, and Crystal RG. Expression of the alpha-1-antitrypsin gene in mononuclear phagocytes of normal and alpha-1-antitrypsin-deficient individuals. *J Clin Invest* 1986;77:1952-1961.
37. Sorensson J, Matejka GL, Ohlson M, and Haraldsson B. Human endothelial cells produce orosomucoid, an important component of the capillary barrier. *Am J Physiol* 1999;276:H530-534.
38. Horadagoda NU, Eckersall PD, Andrew L, Gallay P, Heumann D, and Gibbs HA. Characterisation of bovine lipopolysaccharide binding protein and the in vivo acute phase response to *Pasteurella haemolytica* Type A. *Vet Immunol Immunopathol* 1995;49:61-74.
39. Lamping N, Dettmer R, Schroder NW, Pfeil D, Hallatschek W, Burger R, and Schumann RR. LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria. *J Clin Invest* 1998;101:2065-2071.
40. Nelson S, Bagby CJ, Bainton BG, Wilson LA, Thompson JJ, and Summer WR. Compartmentalization of intraalveolar and systemic lipopolysaccharide-induced tumor necrosis factor and the pulmonary inflammatory response. *J Infect Dis* 1989;159:189-194.
41. Lengacher S, Jongeneel CV, Le Roy D, Lee JD, Kravchenko V, Ulevitch RJ, Glauser MP, and Heumann D. Reactivity of murine and human recombinant LPS-binding protein (LBP) within LPS and gram negative bacteria. *J Inflamm* 1995;47:165-172.
42. Grunwald U, Fan X, Jack RS, Workalemahu G, Kallies A, Stelter F, and Schutt C. Monocytes can phagocytose Gram-negative bacteria by a CD14-dependent mechanism. *J Immunol* 1996;157:4119-4125.
43. Vreugdenhil AC, Snoek AM, van 't Veer C, Greve JW, and Buurman WA. LPS-binding protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction. *J Clin Invest* 2001;107:225-234.
44. Wurfel MM, Kunitake ST, Lichenstein H, Kane JP, and Wright SD. Lipopolysaccharide (LPS)-binding protein is carried on lipoproteins and acts as a cofactor in the neutralization of LPS. *J Exp Med* 1994;180:1025-1035.
45. Laine E, Couderc R, Roch-Arveiller M, Vasson MP, Giroud JP, and Raichvarg D. Modulation of human polymorphonuclear neutrophil functions by alpha 1-acid glycoprotein. *Inflammation* 1990;14:1-9.
46. Boutten A, Dehoux M, Deschenes M, Rouzeau JD, Bories PN, and Durand G. Alpha 1-acid glycoprotein potentiates lipopolysaccharide-induced secretion of interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha by human monocytes and alveolar and peritoneal macrophages. *Eur J Immunol* 1992;22:2687-2695.
47. Bories PN, Feger J, Benbernou N, Rouzeau JD, Agneray J, and Durand G. Prevalence of tri- and tetraantennary glycans of human alpha 1-acid glycoprotein in release of macrophage inhibitor of interleukin-1 activity. *Inflammation* 1990;14:315-323.
48. Muchitsch EM, Varadi K, and Pichler L. Effects of alpha 1-acid glycoprotein on acute pancreatitis and acute lung injury in rats. *Arzneimittelforschung* 2000;50:987-994.
49. Hocheppied T, Van Molle W, Berger FG, Baumann H, and Libert C. Involvement of the acute phase protein alpha 1-acid glycoprotein in nonspecific resistance to a lethal gram-negative infection. *J Biol Chem* 2000;275:14903-14909.
50. de Haas CJ. New insights into the role of serum amyloid P component, a novel lipopolysaccharide-binding protein. *FEMS Immunol Med Microbiol* 1999;26:197-202.
51. de Haas CJ, van der Zee R, Benaissa-Trouw B, van Kessel KP, Verhoef J, and van Strijp JA. Lipopolysaccharide (LPS)-binding synthetic peptides derived from serum amyloid P component neutralize LPS. *Infect Immun* 1999;67:2790-2796.

CHAPTER 9

Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology

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ABSTRACT

Lipopolysaccharide (LPS), a major proinflammatory glycolipid component of the Gram-negative bacterial cell wall, is one of the agents ubiquitously present as a contaminant on airborne particles, including air pollution, organic dusts, and cigarette smoke. Chronic exposure to significant levels of LPS is reported to be associated with the development and/or progression of many types of lung diseases, including asthma, chronic bronchitis, and progressive irreversible airflow obstruction, that are all characterized by chronic inflammatory processes in the lung. In the present study, pathologic effects of long-term LPS exposure to the lung were investigated in detail. To this end, a murine model in which mice were exposed to repeated intratracheal instillation of *Escherichia coli* LPS was developed. We show that long-term LPS instillation in mice results in persistent chronic pulmonary inflammation, characterized by peribronchial and perivascular lymphocytic aggregates (CD4+, CD8+, and CD19+), parenchymal accumulation of macrophages and CD8+ T cells, and altered cytokine expression. Furthermore, airway and alveolar alterations such as mucus cell metaplasia, airway wall thickening, and irreversible alveolar enlargement accompanied the chronic inflammatory response. Interestingly, the observed inflammatory and pathologic changes mimic changes observed in human subjects with chronic inflammatory lung diseases, especially chronic obstructive pulmonary disease (COPD), suggesting that this murine model could be applicable to dissect the role of inflammation in the pathogenesis of these disease conditions.

INTRODUCTION

The respiratory system is continuously exposed to the external environment via the air inhaled, which contains numerous potentially harmful agents. Lipopolysaccharide (LPS), a major proinflammatory glycolipid component of the Gram-negative bacterial cell wall, is one of the agents ubiquitously present as contaminant on airborne particles, including air pollution¹, organic dusts^{2,3}, and cigarette smoke⁴. In ambient breathing air, LPS is measured at very low concentrations ($\pm 0.4 \text{ ng/m}^3$)⁵. Under these conditions, the lung has efficient defense mechanisms against airborne LPS, including the mucociliary system, antimicrobial compounds in the epithelial lining fluid, and alveolar macrophages. Experimental inhalation of a high LPS dose, however, provokes the innate immune system in healthy human subjects, resulting in an acute inflammatory response, which manifests itself at both the pulmonary and the systemic level. In addition, this response is accompanied by clinical symptoms, including fever and airflow decline^{6,7}. Extensive studies investigating acute inflammation using laboratory animals have demonstrated that LPS activates alveolar macrophages via LPS-binding protein (LBP)/CD14/Toll-like receptor (TLR)-4-dependent pathway to produce specific cytokines, resulting in a rapid but transient neutrophil infiltration into the lung (interstitium, alveoli, and airway)⁸.

In contrast to short-term LPS exposure, chronic exposure to significant levels of LPS is reported to be associated with the development and/or progression of many types of lung diseases, including asthma, chronic bronchitis, and progressive irreversible airflow obstruction, all characterized by chronic inflammatory processes in the lung. Michel and colleagues reported that the concentration of LPS in the domestic setting is associated with the clinical severity of asthma⁹. Moreover, individuals with asthma¹⁰ and those with chronic bronchitis¹¹ develop airflow obstruction at lower concentrations of inhaled LPS compared with healthy subjects, and thus respond more sensitively to LPS. Chronic occupational exposure to LPS contained in organic dusts, such as grain dust and swine dust, is known to result in airflow obstruction among agricultural workers. In addition, the concentration of inhaled LPS (not dust) in the workplace bioaerosol is strongly and consistently related to respiratory symptoms and airflow obstruction^{12,13}. Furthermore, follow-up studies have shown that long-term average exposure to LPS-laden dusts resulted in a profound pulmonary inflammatory response related to longitudinal decline in lung function¹⁴, and thus irreversible and progressive lung pathology. Recently, bioactive LPS is reported to be present at high levels in cigarette smoke⁴, which suggests that LPS is an important pathogenic substance in cigarette smoke contributing to the pulmonary diseases that develop in susceptible cigarette smokers. Taken together, these studies indicate that LPS may have a larger role in the pathogenesis of chronic lung diseases than previously realized. However, the extent to which inflammatory processes contribute to lung pathology observed in the lungs of these patients is still poorly understood.

In the present study, pathologic effects of long-term LPS exposure to the lung were investigated in detail. To this end, we developed a murine model in which

mice were exposed to multiple intratracheal instillations of *Escherichia coli* LPS. We show that repeated intratracheal LPS instillation in mice results in persistent chronic pulmonary inflammation with altered cytokine expression, accompanied by airway and alveolar alterations. Interestingly, the observed inflammatory and pathologic changes mimic changes observed in human subjects with chronic pulmonary inflammatory disorders, especially chronic obstructive pulmonary disease (COPD), suggesting that this murine model is applicable to dissect the role of inflammation in the pathogenesis of these disease conditions.

MATERIAL & METHODS

Antibodies

Monoclonal antibody (mAb) Gr-1 (rat anti-mouse Ly-G6) was obtained from PharMingen (San Diego, CA). mAb Moma-2 (rat anti-mouse macrophage/monocyte) was a kind gift from Prof. G. Kraal (Free University, Amsterdam). mAb 6D5 (rat anti-mouse CD19) was obtained from Serotec (Oxford, UK). Hybridoma cell lines GK1.5 (rat anti-mouse CD4), 53-6.72 (rat anti-mouse CD8), and TIB120 (rat anti-murine major histocompatibility complex (MHC) class II) were obtained from the American Type Culture Collection (Rockville, MD). mAb α -SMA-FITC (fluorescein isothiocyanate-conjugated mouse anti-human smooth muscle actin) was obtained from Sigma Chemical Co. (St. Louis, MO). mAb MIB-1 (mouse anti-human peptide Ki-67) was obtained from Immunotech (Marseille, France). Control mAb GL113 (rat IgG) was a gift from Dr. Savelkoul (Erasmus University, Rotterdam, The Netherlands).

Animals

Male Swiss mice ~ 12 wk old were obtained from Charles River Breeding Laboratories (Heidelberg, Germany). Animals were housed individually in standard laboratory cages and allowed food and water *ad libitum* throughout the experiments. The studies were performed under a protocol approved by the Institutional Animal Care Committee of the Maastricht University.

Experimental protocol

Mice were repeatedly challenged with LPS (*E. coli*, serotype O55:B5, Sigma Chemical Co.) twice a week for a period of 12 wk by intratracheal instillation in an attempt to induce a chronic pulmonary inflammation. The dose of LPS used was 5 μ g/instillation/mouse, which corresponds to the LPS dose delivered to the human lung by smoking of ~ 25 cigarettes⁴. No signs of an overall toxic effect at the dose of LPS employed were observed in the trachea, airways, and lungs. Sham mice were instilled intratracheally with LPS-free sterile 0.9% NaCl, whereas control mice received no treatment. Intratracheal instillation was performed by a nonsurgical technique as described previously⁸. In brief, mice were anesthetized by intraperitoneal injection of xylazin/ketamin. A volume of 50 μ l was instilled intratracheally via a canule, followed by 0.1 ml of air. After intratracheal treatment,

the mice were kept in an upright position for 10 min to allow sufficient spreading of the fluid throughout the lungs. Thirty mice were divided into four experimental groups. LPS+1 group ($n = 10$): intratracheal exposure to $5 \mu\text{g}$ LPS; mice were killed 1 wk after the final intratracheal instillation (1-wk recovery period) to allow acute inflammatory changes that occur after each LPS exposure to subside. LPS+8 group ($n = 5$): intratracheal exposure to $5 \mu\text{g}$ LPS; mice were killed 8 wk after the final intratracheal instillation (8-wk recovery period) to study the ongoing character of the induced pathogenesis. Saline group ($n = 10$): intratracheal instillation of $50 \mu\text{l}$ sterile saline; mice were killed 1 wk after the final intratracheal instillation. Control group ($n = 5$): age-matched control mice.

Histologic analysis

After thoracotomy, the left lung was prepared for light microscopy. Lung tissue was inflated with 10% phosphate-buffered formalin ($\text{pH} = 7.4$) at a pressure of 20 cm H_2O through the trachea for 15 min and subsequently fixed in formalin for 24 h. After paraffin embedding, $4 \mu\text{m}$ sections were cut and stained with hematoxylin/eosin (H&E) to evaluate general morphology; or periodic acid-Schiff (PAS)/Alcian blue to determine the presence of mucin glycoconjugates.

Immunohistochemistry

Inflammatory cells were characterized by immunohistochemistry on frozen sections. Lung tissue of the right lung was infused with 20% sucrose/50% TissueTek OCT compound (Sakura Finetek Europe BV; Zoeterwoude, The Netherlands) and slowly frozen in TissueTek OCT on dry ice. Consecutive sections of $5\text{--}7 \mu\text{m}$ thickness were cut, fixed in acetone, and stored at 20°C until use. Sections were rehydrated in tris-buffered saline, $\text{pH} 7.8$, and endogenous peroxidase was quenched with 0.3% H_2O_2 . To prevent nonspecific binding of antibodies, sections were blocked with 5% normal goat serum. Sections were stained with mAbs for detection of neutrophils (Gr-1), macrophages (Moma-2), CD4^+ T-lymphocytes (GK1.5), CD8^+ T-lymphocytes (53-6.72), B-lymphocytes (6D5), and cells expressing MHC class II antigen (TIB120). After washing, peroxidase-conjugated goat anti-rat IgG Ab (Jackson, West Grove, PA) preincubated with 5% normal mouse serum was applied as the secondary detection Ab. Enzymatic reactivity was visualized with 3-amino-9-ethylcarbazole (AEC). Sections were counterstained with hematoxylin and mounted. No significant staining was detected in slides incubated with control mAb (GL113) instead of the primary detecting mAb, indicating the absence of significant background staining.

Immunohistochemistry for α -smooth muscle actin (α -SMA) was performed as described previously¹⁵. In brief, $4 \mu\text{m}$ paraffin sections were deparaffinized and endogenous peroxidase was quenched with 0.3% H_2O_2 in 100% methanol. α -SMA was detected using FITC-labeled α -SMA mAb followed by peroxidase-labeled anti-FITC pAb (Boehringer Mannheim BV, Almere, The Netherlands), and developed with the chromogen 3,3'-diaminobenzidine. Sections were counterstained with hematoxylin and mounted.

Proliferating cells were detected by immunohistochemistry on 4 μm paraffin sections using SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany). After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 100% methanol. Antigen was unmasked by heating the sections for 15 min in 10 mM citrate buffer pH 6.0 using a microwave, followed by 60 min cooling in the same buffer. Proliferating cells were detected by incubating the sections with mAb Ki-67 (MIB-1) for 30 min at 37°C. M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA) was used according to the manufacturer's instructions. The M.O.M. kit significantly reduced background staining caused by the inability of the anti-mouse secondary antibody to distinguish between the mouse primary antibody and endogenous mouse immunoglobulins in the tissue. The peroxidase-labeled secondary antibody was visualized with AEC. Sections were counterstained with hematoxylin and mounted.

Morphologic and morphometric analyses

Lymphocytic aggregates (cutoff value > 50 lymphocytes/aggregate) were counted on H&E-stained lung sections in the hilar region (magnification $\times 50$) by two independent observers unaware of the experimental conditions. Results are expressed as the number of aggregates/section (corrected for surface area).

Intra-alveolar macrophages recognized by general morphology on H&E-stained paraffin sections were enumerated for each section on ten randomly chosen fields (divided into 10×10 squares), which represents a total area of 2.5 mm^2 . This area was sufficient to obtain a mean value per subject that remained rather constant after further increase of the number of fields examined. Intra-alveolar macrophages were counted at a magnification of $\times 200$ by two independent observers unaware of the experimental conditions. Results are expressed as the number of macrophages/ mm^2 .

Lymphocyte subsets recognized by specific immunohistochemical staining on frozen sections were scored on a 4-point scale by one independent observer unaware of the experimental conditions (0 = 0-2 cells; 1 = 3-6 cells; 3 = 7-10 cells; 4 = >10 cells per field). Positive cells were counted in four representative fields by high-power field magnification ($\times 200$).

Airway wall thickening was determined using standard morphometric technique on α -SMA-stained paraffin section cut from the upper part of the left lung. Conducting airways (width > 190 μm) were captured at $\times 20$ with a digital camera and the smooth muscle cell area surrounding the airways was quantified by computerized morphometry using the Quantimet 570 C imaging analysis system (Leica Microsystems, Cambridge, UK). Increased width of the smooth muscle layer was taken as evidence of airway wall thickening.

Standard morphometric technique was used to determine the presence of emphysematous changes in the lungs. In brief, H&E-stained paraffin sections cut from the upper part of the left lung were used, and 10 randomly selected fields were sampled by projecting a microscopic image of the lung section on a screen with a square reference lattice containing one horizontally and one vertically placed test line. The number of intersections of alveolar walls on the test lines

were quantified by computerized morphometry using the Quantimet 570 C imaging analysis system (Leica Microsystems) and used to quantify alveolar mean linear intercept (L_M , the average distance between alveolar walls). Increased L_M was taken as evidence of alveolar enlargement.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from snap frozen lung tissue of the right lung using a commercially available kit (SV Total RNA Isolation System; Promega, Madison, WI). Total RNA concentration and purity was ascertained by electrophoresis on an ethidium bromide-stained 0.8% agarose gel followed by UV illumination and UV spectrophotometric analysis at wavelengths of 260 and 280 nm. Five micrograms of total RNA was reverse transcribed in a 20 μ l volume using oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase (M-MLVRT; Life Technologies, Paisley, UK) according to the supplier's recommendations. PCR was performed in a 25 μ l reaction volume containing 100 μ M of each dNTP, 200 nM sequence-specific primers, and 0.5 U Taq DNA polymerase (Perkin Elmer/Cetus) during 35-40 cycles under the following conditions: 95°C 30 min, annealing temperature (AT) 45 min, 72°C 30 min. PCR primers used in RT-PCR for interleukin (IL)-6 (318 bp, AT 56°C) were sense primer (5'-TGG GAAATCGTGGAAATGAGA-3') and antisense primer (5'-GAGAGCATTGGAAATT GGGGT-3'), whereas PCR primers for tumor necrosis factor (TNF)- α (307 bp, AT 63°C), interferon (IFN)- γ (243 bp, AT 63°C), IL-18 (582 bp, AT 63°C), and β -actin (348 bp, AT 60°C) were designed as previously described^{16,17}. A mock PCR (without cDNA) was included to exclude contamination. cDNA samples were standardized based on the content of β -actin cDNA as housekeeping gene. β -Actin cDNA was evaluated by performance of a β -actin PCR on multiple dilutions of each cDNA sample. The amount of amplified product was estimated by densitometry of gelstar (FMC Bioproducts, Rockland, ME) stained 1.2% agarose gels using a CCD camera and Imagemaster VDS software (Pharmacia). Relative mRNA levels for TNF- α , IL-6, IFN- γ , and IL-18 were calculated by comparison of band intensities of the RT-PCR products to standard curves prepared by PCR amplifications on dilution series of a highly concentrated murine lung cDNA.

Statistical analysis

Statistical analysis was performed by means of one-way ANOVA, and the Mann-Whitney U test was used as post hoc test (Statistical Package for the Social Sciences, version 7.5 for Windows; SPSS Inc., Chicago, IL). Data are expressed as mean $\pm$ standard error of the mean (SEM). A P value < 0.05 denotes the presence of a significant statistical difference.

RESULTS

Persistent chronic pulmonary inflammation after long-term LPS exposure

Histological examination of H&E-stained lung tissue sections of mice repeatedly

Figure 1. Persistent pulmonary inflammation induced by long-term intratracheal LPS instillation. Mice were repeatedly instilled with 5 μ g LPS for 12 weeks, and killed 1 wk [LPS+1] or 8 wk [LPS+8] after the final intratracheal instillation. Age-matched control mice were repeated exposed to saline [S] for 12 wk or not treated [C]. (A) H&E staining of paraffin sections demonstrated persistent peribronchial and perivascular lymphocytic infiltrates (arrows) in LPS-challenged mice and the lack of these inflammatory patterns in control mice. Bar = 200 μ m. (B) Quantification of lymphocytic aggregates, expressed as aggregates/section (mean $\pm$ SEM, $n=5$ to 10 mice/group). * $P<0.05$ versus control; NS, not significant. (C) Higher-power view of H&E-stained paraffin section demonstrating accumulation of macrophages (arrows) in parenchyma of LPS-treated mice. Bar = 50 μ m. (D) Quantitative distribution of intra-alveolar macrophages, expressed as macrophages/mm² (mean $\pm$ SEM, $n=5$ mice/group). * $P<0.05$ versus control; # $P<0.05$ versus LPS+1.

exposed to LPS (5 μ g/mouse) twice/wk for 12 wk demonstrated a striking inflammatory pattern after a 1-wk recovery period. First, lungs of LPS-challenged mice contained dense lymphocytic aggregates, which were observed ubiquitously in all parts of the lungs (Figures 1A and 1B). As illustrated in Figure 1A, aggregates appeared to be located around the airways (peribronchial and peribronchiolar) and large vessels (perivascular), usually without affecting the ciliated bronchial epithelial cells. Incidentally, lymphocytic cells infiltrated the bronchial epithelial layer, resulting in local bronchiolitis (data not shown). These lymphocytic aggregates or infiltrates were not noted in lungs of saline-treated and age-matched control animals. Second, a profound intra-alveolar accumulation of mononuclear leukocytes was clearly noticeable in parenchyma of LPS-exposed mice, which consisted predominantly of macrophages (Figures 1C and 1D). These inflammatory changes were not observed in the parenchyma of saline-treated and age-matched control mice. Interestingly, pulmonary infiltration of neutrophils observed to occur rapidly after each LPS exposure (data not shown) was not observed after 1-wk recovery period, indicating that acute inflammatory changes

were resolved at this time point. To study if LPS-induced pulmonary inflammation is an ongoing process, mice were killed 8 wk after cessation of LPS challenge. Histologic examination demonstrated that lymphocytic aggregates observed at 1 wk after the final LPS exposure were still present after this long recovery period, and appeared to be of similar size and density (Figure 1A-B), suggesting persistence of the inflammatory reaction. Furthermore, parenchymal tissue of LPS-exposed mice showed a significant increase of macrophages at this time point when compared with control mice (Figures 1C and 1D). However, macrophage numbers tended to be slightly but significantly reduced when compared with LPS-treated animals after 1-wk recovery period. Taken together, these data demonstrate that repeated intratracheal instillations of LPS result in a persistent chronic pulmonary inflammatory response, characterized by lymphocytic aggregates around airways and vessels and parenchymal accumulation of mononuclear leukocytes.

Characterization of cellular infiltrates

The specific nature of cellular infiltrates in airways and parenchyma was further characterized by immunohistochemistry on frozen lung sections. To this end, a panel of monoclonal antibodies against murine T lymphocytes (CD4+ or CD8+), B lymphocytes, macrophages, and neutrophils was used. As shown in Figure 2A,

Figure 2. Characterization of cellular infiltrates in LPS-challenged mice. Frozen lung sections of LPS-challenged mice after 1-wk recovery period were evaluated by immunohistochemistry using mAbs for macrophages (Mφ), T lymphocytes (CD4+ or CD8+), and B lymphocytes (CD19+) (A) Composition of lymphocytic aggregates (circle). Note the presence of lymphocytes (CD4+, CD8+, and CD19+), and absence of macrophages in these aggregates. (B) Cellular infiltrates in parenchyma. Note the accumulation of macrophages and CD8+ T cells in the parenchymal area. (C) Expression of MHC class II antigen. Note the low staining for MHC II in control animals [C], and the intense staining for MHC II in intra-alveolar mononuclear leukocytes as well as lymphocytic cells in the aggregates (circle) in LPS-treated animals after 1-wk recovery period [LPS+1]. Bar = 50 μ m. Results are representative for three separate experiments.

Table 1. Lymphocyte subsets in lungs of LPS-challenged mice and controls*

Score	Control (n=5)	Saline (n=5)	LPS+1 (n=5)	LPS+8 (n=4)	p Value
CD4	0.60 ± 0.24	0.60 ± 0.40	0.80 ± 0.37	0.75 ± 0.48	NS
CD8	0.40 ± 0.24	0.60 ± 0.24	2.00 ± 0.45 ¹	2.25 ± 0.48 ¹	< 0.01
CD20	0.40 ± 0.24	0.40 ± 0.40	0.60 ± 0.40	0.75 ± 0.48	NS

* Data are expressed as mean ± SEM; NS = not significant; ¹ Significant versus saline-treated and control animals.

immunohistochemical staining identified CD4+ T lymphocytes as the principal component of the aggregates present in lungs of LPS-exposed mice after a short recovery period. Other mononuclear cells included CD8+ T lymphocytes and B lymphocytes, which were present to lesser extent at the periphery of these aggregates. Thorough examination of serial sections revealed that aggregates were not organized in T cell and B cell zones, thus lacking any resemblance with lymph nodes or bronchus-associated lymphoid tissue (BALT). In addition, neither neutrophils nor macrophages were noted in these aggregates. As shown in Figure 2B, immunohistochemical staining confirmed the prominent accumulation of macrophages as well as the absence of infiltrated neutrophils in the parenchyma of LPS-challenged mice. In addition, positive staining for lymphocytes was observed in the alveolar area of mice repeatedly exposed to LPS; this was not clearly noticeable in H&E-stained paraffin sections. Interestingly, the number of CD8+ T cells, but not CD4+ T cells and CD19+ B cells, in the parenchyma was significantly increased relative to controls (Table 1). Furthermore, evaluation lungs from LPS-exposed mice killed after an 8-wk recovery period demonstrated that cessation of LPS challenge did not result in an altered composition of the lymphocytic aggregates and the inflammatory pattern in the parenchyma (Table 1). Additionally, we analyzed MHC class II antigen expression, which is considered to be a marker for cell activation that correlates with the intensity of the immune response¹⁸. As shown in Figure 2C, low staining for MHC II was noted in alveolar macrophages from control animals. In LPS-challenged animals, mononuclear leukocytes present in the parenchyma as well as lymphocytic cells located in the aggregates displayed positive staining for MHC II after 1-wk (Figure 2C) and 8-wk (data not shown) recovery periods. The staining intensity was strongly increased in LPS-challenged mice when compared with controls, indicating that infiltrated inflammatory cells in both airway and parenchymal regions were activated. Taken together, these data demonstrated CD4+ T cells to be the principal component of peribronchial and perivascular lymphocytic aggregates observed after 1-wk and 8-wk recovery periods, whereas macrophages and CD8+ T cells were identified to accumulate in the parenchymal area of LPS-exposed mice. Furthermore, infiltrated inflammatory cells were activated in respect of MHC II expression.

Cytokine mRNA expression

Because the chronic character of an inflammatory response is possibly due to

alteration in expression levels of Th1 cytokines¹⁹⁻²¹, we investigated mRNA levels of the Th1 type cytokines TNF- α , IL-6, and IFN- γ in lungs of LPS-exposed mice and controls using RT-PCR. Additionally, IL-18 mRNA levels were determined, which is known to be involved in IFN- γ expression and to be associated with chronic inflammation. After correction for β -actin levels, lungs of LPS-exposed mice demonstrated enhanced mRNA expression for TNF- α , IFN- γ , and IL-18, whereas these mRNA levels were low or negligible in saline-exposed and noninstilled lungs (Figure 3A). Remarkably, expression levels for all three cytokines further increased after 8 wk when compared with levels after a 1-wk recovery period. In contrast, lungs of saline-exposed and control mice demonstrated constitutive mRNA levels for IL-6, but these levels were not increased after long-term LPS exposure. These observations were confirmed by the calculation of the densitometric ratio for each mRNA expression (Figure 3B).

Figure 3. Transcription of inflammatory mediators in murine lung tissue as determined by RT-PCR. Total RNA was isolated from lung tissue of age-matched control mice [C], saline-treated mice [S], and LPS-challenged mice after 1-wk [LPS+1] and 8-wk [LPS+8] recovery period. (A) mRNA expression levels of TNF- α (307 bp), IL-6 (318

bp), IFN- γ (243 bp), IL-18 (582 bp) and β -actin (348 bp) were analyzed using RT-PCR. Equal intensity of β -actin mRNA bands confirmed equal loading of cDNA in RT-PCR. One representative experiment from a series of three is shown. (B) Densitometric ratio's ($n=3$ mice/group).

Airway and alveolar alterations after long-term LPS exposure

Lungs of LPS-challenged mice demonstrated a variety of abnormalities in airways and parenchyma. First, lung sections stained with PAS/Alcian blue showed a marked increase of mucus producing cells numbers after a 1-wk recovery period, compared with saline-treated controls (Figure 4A). Metaplasia of airway goblet cell was evident in the larger airways of the respiratory tract, especially at sites where lymphocytic infiltrates were underlying the bronchial epithelium. Secretory cell metaplasia was also observed 8 wk after cessation of LPS challenge, but to a lesser extent, suggesting that these metaplastic events may be reversible. Second, airway walls of LPS-exposed mice were thickened when compared with controls. Immunohistochemical staining indicated the presence of α -SMA in small, elongated cells arranged circumferentially in a single layer in the airway submucosa of control mice. On the other hand, airways of LPS-challenged mice were characterized by a patchy α -SMA staining, and commonly of two layers of nuclei in the airway submucosa. As shown in Figure 4B, width of the smooth muscle layer was significantly increased after both 1-wk and 8-wk recovery periods compared with controls. Alveolar alterations were examined by morphometric measurements of alveolar enlargement and evaluation of

proliferation. Morphometric evaluation of alveolar dimensions revealed emphysematous changes in LPS-challenged mice at 1-wk recovery period (Figures 4C and 4D). In contrast, repeated saline exposure did not result in alveolar enlargement when compared with controls. Importantly, lungs from LPS-exposed mice sacrificed after an 8-wk recovery period still displayed emphysematous changes, indicating permanent enlargement induced by long-term LPS exposure. Furthermore, the expression of Ki-67 antigen, a marker of active cell proliferation, was evaluated to investigate presence of repair processes. Alveolar epithelial cells of LPS-exposed mice did not display Ki-67 staining, neither after 1-wk nor after 8-wk recovery periods (data not shown), indicating the absence of active repair processes. Taken together, these data show that long-term LPS exposure results in pathologic changes in airways and parenchyma, characterized by goblet cell metaplasia and airway wall thickening, and irreversible alveolar enlargement.

Figure 4. Airway and alveolar abnormalities after long-term intratracheal LPS instillation. (A) Mucus cell metaplasia. Representative lung histology (PAS/Alcain blue staining) of mice repeatedly exposed to saline [S] or LPS after 1-wk recovery period [LPS+1]. Note the mucus producing bronchial epithelial cells in LPS-challenged mice (arrows). Bar = 25 μm . (B) Morphometric analysis of airway wall thickening. Paraffin sections were stained for α -SMA, and width of smooth muscle layer is expressed in μm . * $P < 0.05$ versus control; NS, not significant. (C) Alveolar enlargement. H&E staining of paraffin sections demonstrated destruction of alveolar walls in LPS-instilled mice after 1-wk recovery period [LPS+1] compared to control mice [C]. Bar = 100 μm . (D) Morphometric analysis of alveolar enlargement. Alveolar mean linear intercept (L_M) values are expressed in μm (mean $\pm$ SEM, $n = 5$ mice/group). * $P < 0.05$ versus control; NS, not significant.

DISCUSSION

This study investigated inflammatory and pathologic alterations after long-term LPS exposure to the lung. Mice were repeatedly exposed to LPS and killed after 1-wk or 8-wk recovery periods. We have previously demonstrated that single intratracheal LPS challenge results in rapid but transient infiltration of neutrophils

from the pulmonary vascular bed into the respiratory air spaces⁸ associated with acute lung injury, such as increased pulmonary vascular permeability and pulmonary edema. The present study is the first to characterize chronic inflammatory events induced by repeated intratracheal LPS instillation. We demonstrate that long-term intratracheal LPS exposure resulted in a persistent chronic inflammatory reaction, which is accompanied by alterations in airways and parenchyma.

Lungs of LPS-challenged mice contained dense peribronchial and perivascular lymphocytic aggregates, which did not appear to be organized in lymphoid follicles. In addition, aggregates were shown to consist of mainly T lymphocytes and less B lymphocytes (no macrophages) supporting the observation that these lymphocytic aggregates show no similarity with normal lymph nodes or BALT present in the murine lung²². Origin of these aggregates is uncertain, but they may originate from small T and B cell clusters that have been described in the murine lung in the vicinity of bronchioles and veins²². Interestingly, this type of mononuclear inflammatory cell accumulation in the peribronchial area was also noted in transgenic mice overexpressing specific proinflammatory cytokines in the airway epithelial cells^{20,23,24}. Especially peribronchial infiltrates in human IL-6 transgenic mice show striking similarity to our observations, e.g., significant numbers of CD4+ and CD8+ T lymphocytes and B lymphocytes, but lacking alveolar macrophages²⁰.

Long-term LPS exposure also resulted in a chronic inflammatory response in the parenchymal area, which was comprised of activated macrophages indicated by increased expression of MHC class II molecules on the cell surface and CD8+ T cells. The absence of neutrophils at 1 wk after the last LPS exposure indicates that neutrophil infiltration is rather a transient process triggered by each LPS exposure instead of being a chronic factor. Recently, Ofulue and colleagues demonstrated that long-term exposure (up to 6 mo) to cigarette smoke in rats resulted in a similar increase of intra-alveolar macrophages²⁵. This result is in accordance with our observations, assuming that LPS is an important contaminant of cigarette smoke⁴.

An interesting result of the present study is that the characteristic inflammatory pattern in the lung induced by repeated intratracheal LPS instillation continued after cessation of the LPS challenge, which supports the important role for LPS in chronic lung inflammation. Studies using transgenic mice^{19,21} suggest that altered expression levels of Th1-type cytokines like TNF- α , IL-6, and IFN- γ may contribute to the ongoing inflammatory response and structural changes in airways and parenchyma. We therefore investigated mRNA levels of these specific Th1-type cytokines in lungs of LPS-exposed mice, and also mRNA levels of IL-18, which is involved in IFN- γ expression and has been associated with chronic inflammation. The inflammatory response in the LPS-treated animals was characterized by significant increases in mRNA levels for TNF- α , IL-18, and IFN- γ , which further increased after cessation of LPS challenge. These data support the idea that ongoing inflammatory processes induced by long-term LPS exposure results from an altered cytokine balance, involving cytokines derived from both macrophages

and lymphocytes. Interestingly, long-term LPS exposure did not influence the constitutive mRNA levels for IL-6 in saline-exposed and control lungs. This was unexpected, since the pattern of pulmonary inflammation in IL-6 transgenic mice showed striking similarities with our observations²⁰. However, IL-6 is documented to be a downregulator of LPS-induced TNF- α and IL-1 β expression *in vitro* and a strong inhibitor of LPS-induced acute inflammation in rats^{26,27}. We speculate that the lack of IL-6 upregulation in lungs of mice repeatedly exposed to LPS may contribute to an altered cytokine balance. The exact mechanism by which long-term LPS exposure alters the cytokine balance, however, is unclear and awaits further investigation.

In addition to the persistent inflammatory events, long-term LPS exposure resulted in significant airway and alveolar abnormalities. First, bronchial epithelium of LPS-challenged mice displayed an increase in the mucus-producing goblet cells in the larger airways, especially at sites where lymphocytic infiltrates were underlying the bronchial epithelium. Because only small numbers of goblet cells are present in murine airways, this increase is likely due to cell differentiation (metaplasia) rather than cell division (hyperplasia). In line with our findings, increase of airway mucus cells was reported after single intratracheal LPS instillation in mice²⁸, and repeated intratracheal LPS exposure in rats²⁹ and hamsters³⁰. Whether the mucus cell metaplasia observed by histologic analysis also involves increased expression of mucin genes obviously needs further study. Second, airways of LPS-challenged mice demonstrated thickening of the smooth muscle layer. Recently, George and colleagues³¹ demonstrated similar airway wall thickening with patchy actin staining after long-term exposure to corn dust extract in mice, which is known to contain high levels of LPS. In addition, increased bronchial wall thickness was also demonstrated in transgenic mice overexpressing IL-6 or IL-11 in airway epithelial cells³². Third, long-term intratracheal LPS exposure resulted in alveolar enlargement without any signs of proliferation of alveolar epithelial cells, which was shown to be irreversible. Consistent with our data, previous reports described approximately equal extent of parenchymal destruction in the lungs of hamsters^{30,33} and mice³⁴ after repeated LPS challenge using different instillation protocols, and after long-term exposure to cigarette smoke^{25,35}. However, lungs of transgenic mice overexpressing specific proinflammatory cytokines inducible or not in alveolar type II cells (TNF- α ¹⁹) or airway epithelial cells (IFN- γ ²¹, IL-6, or IL-11³²) displayed enormous alveolar destruction ($L_M > 100\%$).

The pathologic changes we observed in both airways and parenchyma after multiple LPS challenge in mice mimic important features observed in COPD patients, such as goblet cell metaplasia, airway wall thickening, and emphysematous changes. Recent histopathologic studies have demonstrated a prominent infiltration of macrophages and CD8+ lymphocytes in bronchial tissue and lung parenchyma in COPD³⁶, both of which we also observed in the lungs of mice after long-term LPS exposure. Additionally, neutrophil infiltration is shown particularly in the central airways of COPD patients³⁷. The fact that we focused on peripheral airways and parenchyma of LPS-exposed mice may therefore explain

the lack of neutrophils as a chronic factor in our model. Increasing evidence emerges that both macrophages and neutrophils have an important role in the proteolytic degradation of the extracellular matrix in COPD by production of matrix metalloproteinases. The role of CD8+ T cells and their mediators in this process is, however, still uncertain but very intriguing as they may be involved in apoptosis and destruction of alveolar-wall epithelial cells through the release of TNF- α , perforins, and granzymes³⁷.

Pulmonary burden with various reagents may potentially result in a response by the respiratory system. Concerning LPS exposure, numerous studies demonstrated that chronic exposure to significant levels of LPS in man is associated with the development and/or progression of many types of chronic inflammatory lung diseases, including asthma, chronic bronchitis, and progressive irreversible airflow obstruction. Additionally, this study showed that long-term LPS exposure in mice induces a chronic mononuclear inflammatory response with specific pathologic changes mimicking COPD. Because the major environmental factor that predisposes patients to COPD is long-term cigarette smoking, we speculate that LPS present in cigarette smoke is a good candidate to trigger smoking-induced accumulation of macrophages, lymphocytes, and neutrophils in respiratory bronchioles, alveolar ducts, and alveoli in susceptible cigarette smokers. We consider this experimental murine model an important means for further elucidation of the role of chronic inflammation in the pathogenesis of chronic pulmonary disorders like COPD.

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REFERENCES

1. Kline JN, Cowden JD, Hunninghake GW, Schutte BC, Watt JL, Wohlford Lenane CL, Powers LS, Jones MP, and Schwartz DA. Variable airway responsiveness to inhaled lipopolysaccharide. *Am J Respir Crit Care Med* 1999;160:297-303.
2. Rylander R, Haglund P, and Lundholm M. Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom. *Am Rev Respir Dis* 1985;131:209-213.
3. Del A, Godshali MA, and Palmgren MS. Gram-negative bacterial endotoxins in grain elevator dusts. *Am Ind Hyg Assoc J* 1984;45:336-339.
4. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, and Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999;115:829-835.
5. Hartung J, and Seedorf J. Orienting endotoxin measurement in the atmosphere. *DTW Dtsch Tierarztl Wochenschr* 1999;106:522-525.
6. Michel O, Duchateau J, Plat G, Cantinieux B, Hotimsky A, Gerain J, and Sergysels R. Blood inflammatory response to inhaled endotoxin in normal subjects. *Clin Exp Allergy* 1995;25:73-79.
7. Jagielo PJ, Thorne PS, Watt JL, Frees KL, Quinn TJ, and Schwartz DA. Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects. *Chest* 1996;110:263-270.

8. Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA, and Wouters EF. Intratracheal Instillation of Lipopolysaccharide in Mice Induces Apoptosis in Bronchial Epithelial Cells. No role for tumor necrosis factor- α and infiltrating neutrophils. *Am J Respir Cell Mol Biol* 2001;24:569-576.
9. Michel O, Duchateau J, and Sergysels R. Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. *J Appl Physiol* 1989;66:1059-1064.
10. Michel O, Kips J, Duchateau J, Vertongen F, Robert L, Collet H, Pauwels R, and Sergysels R. Severity of asthma is related to endotoxin in house dust. *Am J Respir Crit Care Med* 1996;154:1641-1646.
11. Cavagna G, Foa V, and Vigliani EC. Effects in man and rabbits of inhalation of cotton dust or extracts and purified endotoxins. *Br J Ind Med* 1969;26:314-321.
12. Smid T, Heederik D, Houba R, and Quanjer PH. Dust- and endotoxin-related acute lung function changes and work-related symptoms in workers in the animal feed industry. *Am J Ind Med* 1994;25:877-888.
13. Schwartz DA, Thorne PS, Yagla SJ, Burmeister LF, Olenchock SA, Watt JL, and Quinn TJ. The role of endotoxin in grain dust-induced lung disease. *Am J Respir Crit Care Med* 1995;152:603-608.
14. Vogelzang PF, van der Gulden JW, Folgering H, Kolk JJ, Heederik D, Preller L, Tielen MJ, and van Schayck CP. Endotoxin exposure as a major determinant of lung function decline in pig farmers. *Am J Respir Crit Care Med* 1998;157:15-18.
15. Lutgens E, Daemen M, Kockx M, Doevendans P, Hofker M, Havekes L, Wellens H, and de Muinck ED. Atherosclerosis in APOE*3-Leiden transgenic mice: from proliferative to atheromatous stage. *Circulation* 1999;99:276-283.
16. Daemen MA, van de Ven MW, Heineman E, and Buurman WA. Involvement of endogenous interleukin-10 and tumor necrosis factor- α in renal ischemia-reperfusion injury. *Transplantation* 1999;67:792-800.
17. Daemen MA, van't Veer C, Wolfs TG, and Buurman WA. Ischemia/reperfusion-induced IFN- γ up-regulation: involvement of IL-12 and IL-18. *J Immunol* 1999;162:5506-5510.
18. Otten LA, Steimle V, Bontron S, and Mach B. Quantitative control of MHC class II expression by the transactivator CIITA. *Eur J Immunol* 1998;28:473-478.
19. Fujita M, Shannon JM, Irvin CG, Fagan KA, Cool C, Augustin A, and Mason RJ. Overexpression of tumor necrosis factor- α produces an increase in lung volumes and pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L39-49.
20. DiCosmo BF, Geba GP, Picarella D, Elias JA, Rankin JA, Stripp BR, Whitsett JA, and Flavell RA. Airway epithelial cell expression of interleukin-6 in transgenic mice. Uncoupling of airway inflammation and bronchial hyperreactivity. *J Clin Invest* 1994;94:2028-2035.
21. Wang Z, Zheng T, Zhu Z, Homer RJ, Riese RJ, Chapman HA, Jr., Shapiro SD, and Elias JA. Interferon gamma induction of pulmonary emphysema in the adult murine lung. *J Exp Med* 2000;192:1587-1600.
22. Kolopp Sarda MN, Bene MC, Massin N, Moulin JJ, and Faure GC. Immunohistological analysis of macrophages, B-cells, and T-cells in the mouse lung. *Anat Rec* 1994;239:150-157.
23. Temann UA, Geba GP, Rankin JA, and Flavell RA. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J Exp Med* 1998;188:1307-1320.
24. Tang W, Geba GP, Zheng T, Ray P, Homer RJ, Kuhn C, 3rd, Flavell RA, and Elias JA. Targeted expression of IL-11 in the murine airway causes lymphocytic inflammation, bronchial remodeling, and airways obstruction. *J Clin Invest* 1996;98:2845-2853.
25. Ofulue AF, Ko M, and Abboud RT. Time course of neutrophil and macrophage elastolytic activities in cigarette smoke-induced emphysema. *Am J Physiol* 1998;275:L1134-1144.
26. Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, and Dinarello CA. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. *Blood* 1990;75:40-47.
27. Ulich TR, Yin S, Guo K, Yi ES, Remick D, and del Castillo J. Intratracheal injection of endotoxin and cytokines. II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation. *Am J Pathol* 1991;138:1097-1101.
28. Yanagihara K, Seki M, and Cheng PW. Lipopolysaccharide Induces Mucus Cell Metaplasia in Mouse Lung. *Am J Respir Cell Mol Biol* 2001;24:66-73.

29. Harkema JR, and Hotchkiss JA. Ozone- and endotoxin-induced mucous cell metaplasias in rat airway epithelium: novel animal models to study toxicant-induced epithelial transformation in airways. *Toxicol Lett* 1993;68:251-263.
30. Stolk J, Rudolphus A, Davies P, Osinga D, Dijkman JH, Agarwal L, Keenan KP, Fletcher D, and Kramps JA. Induction of emphysema and bronchial mucus cell hyperplasia by intratracheal instillation of lipopolysaccharide in the hamster. *J Pathol* 1992;167:349-356.
31. George CL, Jin H, Wohlford-Lenane CL, O'Neill ME, Phipps JC, O'Shaughnessy P, Kline JN, Thorne PS, and Schwartz DA. Endotoxin responsiveness and subchronic grain dust-induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L203-213.
32. Kuhn C, 3rd, Homer RJ, Zhu Z, Ward N, Flavell RA, Geba GP, and Elias JA. Airway hyperresponsiveness and airway obstruction in transgenic mice. Morphologic correlates in mice overexpressing interleukin (IL)-11 and IL-6 in the lung. *Am J Respir Cell Mol Biol* 2000;22:289-295.
33. Milton DK, Godleski JJ, Feldman HA, and Greaves IA. Toxicity of intratracheally instilled cotton dust, cellulose, and endotoxin. *Am Rev Respir Dis* 1990;142:184-192.
34. Starcher B, and Williams I. The beige mouse: role of neutrophil elastase in the development of pulmonary emphysema. *Exp Lung Res* 1989;15:785-800.
35. Hautamaki RD, Kobayashi DK, Senior RM, and Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* 1997;277:2002-2004.
36. Saetta M, Baraldo S, Corbino L, Turato G, Braccioni F, Rea F, Cavallero G, Tropeano G, Mapp CE, Maestrelli P, Ciaccia A, and Fabbri LM. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;160:711-717.
37. Barnes PJ. Chronic obstructive pulmonary disease. *N Engl J Med* 2000;343:269-280.

CHAPTER 10

Discussion and future directions

INTRODUCTION

According to the ATS statement in 1995¹, COPD is characterized by the presence of airflow limitation that is generally progressive and not fully reversible. This physiological definition of COPD has definitely restricted the way of thinking about the pathogenesis of this heterogeneous disease, resulting in a lack of knowledge concerning the molecular and cell biology of COPD and few advances in therapy today. According to the new definition developed by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) expert panel in 2001, the airflow limitation in COPD is associated with an abnormal inflammatory response of the lungs to noxious particles of gases². Thus, COPD is increasingly recognized as a chronic inflammatory disorder. The studies described in this thesis were performed to obtain better insight in the chronic inflammatory processes that are suggested to contribute to the pathogenesis of COPD. The specific aims were to investigate the relationship between local and systemic inflammation in COPD patients, and to further characterize the local inflammatory response (Chapter 3-6). In addition, the effects of acute and long-term LPS exposure to the lung *in vivo* were studied using an experimental animal model (Chapter 7-9). In this chapter, the results of the studies presented in this thesis will be considered in the context of the available literature. In addition, future lines of investigation will be discussed.

PULMONARY INFLAMMATION IN COPD

There is increasing evidence that COPD is associated with a chronic inflammatory response throughout the airways, lung parenchyma, and pulmonary vasculature, with certain variations in inflammatory cells in the different compartments of the lung. As outlined in Chapter 1, cellular inflammatory infiltrates and production of inflammatory mediators, proteinases, and reactive nitrogen and oxygen species are all thought to contribute to the pathologic changes characteristic for COPD. Studies described in Chapter 3-5 were performed to further characterize the local inflammatory response in COPD, hereby focusing on cellular infiltrates, chemokines (IL-8), cytokines (TNF- α , leptin), soluble cytokine receptors (sTNF-R55, sTNF-R75), acute-phase reactants (AGP, LBP, CRP). We demonstrate elevated levels of the various types of inflammatory mediators mentioned in induced

TNF- α as potential therapeutic target in RA and IBD required extensive studies of not just cytokine expression, but also cytokine regulation. To this end, *in vitro* (or *ex vivo*) models were used to understand the disease process, and animal models were developed to confirm the *in vitro* findings¹⁶.

Future research of COPD has to run through different stages. First, COPD has to be generally accepted as a chronic inflammatory disease, as worldwide research is necessary to gain more knowledge about all aspects of inflammation in COPD. Second, *in vitro* (*ex vivo*) studies are crucial to elucidate underlying mechanisms of chronic inflammatory processes in COPD, perhaps also in different stages of the disease. Stimulation experiments in cell lines, and primary cultures in particular, are important to understand reactivity of lung cells to noxious particles and gases. In this respect, it is essential to learn more about the persistence of the inflammatory response after smoking cessation. Knowledge from *in vitro* studies will lead to new hypotheses concerning molecular mechanisms and therapeutical targets. Third, these new hypotheses have to be preferentially evaluated in proper animal models of COPD. Last, integration of multiple disciplines like in IBD and RA will accelerate progress in investigations of COPD, providing a more coherent knowledge and understanding of the molecular mediators and mechanisms of chronic inflammation and tissue injury.

COPD MODELING

As indicated above, future investigations should also be extended to animal models, in which the many unsolved issues in COPD, such as mechanisms of airflow obstruction, inflammatory cell recruitment, persistence of chronic inflammation, and abnormal repair, can be studied. In the past, many animal models for emphysema have been developed (Chapter 1). Exogenous administration of proteinases, chemicals, particulates, and exposure to cigarette smoke was demonstrated to result in pathologic features characteristic of human emphysema. In addition, airspace enlargement during development and throughout life was demonstrated in genetic manipulated mice (both gain and loss of function models). To date, however, no satisfactory animal model mimicking both chronic inflammation and pathologic changes in COPD was available that is suitable for early drug testing.

Recently, bioactive LPS was demonstrated to be present at high levels in cigarette smoke³. Since long-term cigarette smoking is the major environmental factor that predisposes patients to COPD, LPS present in cigarette smoke is a good candidate triggering smoking-induced accumulation of inflammatory cells in respiratory bronchioles, alveolar ducts, and alveoli in cigarette smokers susceptible for COPD. In addition, chronic exposure to significant levels of LPS was reported to be associated with the development and/or progression of COPD. Therefore, animal studies were performed to investigate the effects of acute and long-term LPS exposure to the lung *in vivo* in more detail (Chapter 7-9).

Previous studies have demonstrated that single IT LPS challenge results in a

rapid but transient inflammatory response, characterized by infiltration of neutrophils from the pulmonary vascular bed into the respiratory air spaces^{17,18}. In Chapter 7 and 8, we demonstrate that acute exposure of the lung to LPS has broad-spectrum inflammatory effects both locally and systemically, but also triggers rapid tissue injury at the local level.

Of interest in COPD is chronic exposure to LPS. In Chapter 9, we show that long-term LPS exposure results in persistent chronic inflammation in airways and parenchyma with altered cytokine expression, hereby supporting the idea that ongoing inflammatory processes results from an altered cytokine balance, involving cytokines derived from inflammatory cells. In addition, chronic LPS exposure was shown to induce significant airway and alveolar abnormalities. The inflammatory and pathological changes observed in mice exposed to repeated local LPS exposure mimic important features observed in human subjects with COPD. Therefore, this murine model may be applicable to dissect the role of chronic inflammation in the pathogenesis of COPD. Using intervention strategies, the involvement of a single inflammatory mediator or specific inflammatory cells in the process of LPS-induced chronic inflammation can be studied. In addition, the combination of gene targeting (overexpression and knock-out models) with this LPS exposure model will supply information about individual candidate genes that may contribute to the pathology induced by long-term LPS exposure. As both inflammatory and pathological changes were induced by repeated local LPS exposure, this model may also be suitable for early testing of drugs regarding inflammation or proteolytic damage. To this end, however, further characterization of the model will be necessary, including local inflammation and tissue injury, lung function, and systemic inflammation.

CLOSING REMARKS

We are now only at the beginning of better understanding the disease process of COPD. More research concerning chronic inflammation in COPD is clearly necessary to identify therapeutic targets in COPD and effective therapy. *In vitro* studies and animal studies will contribute to our present understanding of lung and airway inflammation, and its involvement in the pathogenesis of COPD. In addition, studies investigating genetic susceptibility are essential to identify genetic factors important in COPD. Better insights into the pathogenesis of COPD will contribute not only to modulation of the disease process, but may direct future early intervention strategies in order to avoid progression to a state of functional impairment and disablement of patients suffering from this disease. Basic insight into the pathogenesis of this disease process will support raising expectations in the management of these patients, in order to attenuate the future burden of COPD worldwide.

REFERENCES

1. ATS. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1995;152:S77-121.
2. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, and Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001;163:1256-1276.
3. Hasday JD, Bascom R, Costa JJ, Fitzgerald T, and Dubin W. Bacterial endotoxin is an active component of cigarette smoke. *Chest* 1999;115:829-835.
4. Anthonisen NR, Connett JE, Kiley JP, Altose MD, Bailey WC, Buist AS, Conway WA, Jr., Enright PL, Kanner RE, O'Hara P, and et al. Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV1. The Lung Health Study. *Jama* 1994;272:1497-1505.
5. Scanlon PD, Connett JE, Waller LA, Altose MD, Bailey WC, and Buist AS. Smoking cessation and lung function in mild-to-moderate chronic obstructive pulmonary disease. The Lung Health Study. *Am J Respir Crit Care Med* 2000;161:381-390.
6. Kanner RE, Connett JE, Williams DE, and Buist AS. Effects of randomized assignment to a smoking cessation intervention and changes in smoking habits on respiratory symptoms in smokers with early chronic obstructive pulmonary disease: the Lung Health Study. *Am J Med* 1999;106:410-416.
7. Mullen JB, Wright JL, Wiggs BR, Pare PD, and Hogg JC. Structure of central airways in current smokers and ex-smokers with and without mucus hypersecretion: relationship to lung function. *Thorax* 1987;42:843-848.
8. Turato G, Di Stefano A, Maestrelli P, Mapp CE, Ruggieri MP, Roggeri A, Fabbri LM, and Saetta M. Effect of smoking cessation on airway inflammation in chronic bronchitis. *Am J Respir Crit Care Med* 1995;152:1262-1267.
9. Yamamoto C, Yoneda T, Yoshikawa M, Fu A, Tokuyama T, Tsukaguchi K, and Narita N. Airway inflammation in COPD assessed by sputum levels of interleukin-8. *Chest* 1997;112:505-510.
10. Rutgers SR, Postma DS, ten Hacken NH, Kauffman HF, van Der Mark TW, Koeter GH, and Timens W. Ongoing airway inflammation in patients with COPD who do not currently smoke. *Thorax* 2000;55:12-18.
11. Willemse BW, Ten Hacken NH, Rutgers B, G. Li, Postma DS, and Timens W. The effect of one year smoking cessation on neutrophilic inflammation in smokers with and without COPD. *Am J Respir Crit Care Med* 2002;165:A463.
12. Farrell RJ, and Peppercorn MA. Ulcerative colitis. *Lancet* 2002;359:331-340.
13. Shanahan F. Crohn's disease. *Lancet* 2002;359:62-69.
14. Keating GM, and Perry CM. Infliximab: an updated review of its use in Crohn's disease and rheumatoid arthritis. *BioDrugs* 2002;16:111-148.
15. Abuzakouk M, Feighery C, and Jackson J. Tumour necrosis factor blocking agents: a new therapeutic modality for inflammatory disorders. *Br J Biomed Sci* 2002;59:173-179.
16. Feldmann M, and Maini RN. Discovery of TNF-alpha as a therapeutic target in rheumatoid arthritis: preclinical and clinical studies. *Joint Bone Spine* 2002;69:12-18.
17. Hirano S. Quantitative time-course profiles of bronchoalveolar lavage cells following intratracheal instillation of lipopolysaccharide in mice. *Ind Health* 1997;35:353-358.
18. Ulich TR, Yin S, Guo K, Yi ES, Remick D, and del Castillo J. Intratracheal injection of endotoxin and cytokines. II. Interleukin-6 and transforming growth factor beta inhibit acute inflammation. *Am J Pathol* 1991;138:1097-1101.

CHAPTER 11

Summary

INTRODUCTION

In the present thesis, the following topics were studied:

- The relationship between local and systemic inflammation in COPD patients, and further characterization the local inflammatory response;
- The effects of acute and long-term LPS exposure to the lung *in vivo* in an experimental animal model.

Below, the results of the studies on these topics are shortly summarized.

THE RELATIONSHIP BETWEEN LOCAL AND SYSTEMIC INFLAMMATION IN COPD PATIENTS

Chronic inflammation is an important feature of COPD. This inflammatory response is not restricted to the local compartment – including airways, lung parenchyma, and pulmonary vasculature – but is also present in the circulation. However, the origin of the systemic inflammation present in COPD patients remains still to be elucidated. In Chapter 3 and 4, we investigated the relationship between the local inflammatory response in the respiratory organ and systemic inflammation in patients with mild-to-moderate COPD. We assessed airway inflammation using induction of sputum by inhalation of hypertonic saline, which is a safe, non-invasive, and reproducible method to determine cell counts and protein concentrations in the airway compartment of subjects.

In Chapter 3, levels of sTNF-receptors, TNF- α , and IL-8 in induced sputum and plasma were compared between COPD patients and healthy smokers. COPD patients appeared to have significantly higher percentages of neutrophils and levels of sTNF-receptors and IL-8 in induced sputum as compared to healthy smokers. These parameters highly correlated with each other and with the degree of airflow limitation. In contrast, sputum TNF- α levels were similar in both groups. When comparing levels of inflammatory mediators in sputum and plasma, no direct correlations were found. In addition, sputum levels of sTNF-receptors were related with degree of airflow limitation in the COPD group, whereas plasma levels were not. These observations suggests that the systemic inflammatory response in COPD is not due to an overflow of inflammatory mediators from the local compartment, but that the inflammatory processes in the local and systemic compartment are differently regulated.

In Chapter 4, levels of three major acute-phase reactants –AGP, CRP, and LBP– were assessed in induced sputum and plasma of COPD patients and healthy smokers. Sputum levels of AGP and LBP were significantly increased in COPD patients versus healthy smokers, and inversely correlated with the FEV₁ values in COPD. In contrast, sputum CRP was not different between the study groups. In plasma, LBP was markedly elevated in COPD patients versus healthy smokers, whereas circulating levels of AGP and CRP tended to be increased in COPD. In contrast to sputum, AGP and LBP in plasma were positively related to the FEV₁ values. Exploring the relationship between local and systemic acute-phase response, no direct relations, which suggests that acute-phase reactants in sputum may be due to local production rather than leakage from the systemic compartment.

FURTHER CHARACTERIZATION THE LOCAL INFLAMMATORY RESPONSE IN COPD PATIENTS

Increased levels of several cytokines and chemokines have been found in association with COPD. However, overall fundamental questions regarding the specific role inflammatory mediators and mechanisms of inflammatory cell recruitment in COPD are still remaining because our knowledge about chronic inflammation is still fragmentary. In order to get insight in the underlying mechanisms of the chronic inflammation in COPD, we further characterized the local inflammatory response.

As indicated above, COPD patients had levels of sTNF-receptors, in particular sTNF-R55, in induced sputum as compared to healthy smokers (Chapter 3). In addition, increased levels of AGP and LBP were demonstrated in COPD sputum as compared to controls (Chapter 4). Our data on smoking cessation (Chapter 3) suggest an ongoing inflammation in airways and circulation of COPD patients after smoking cessation.

Leptin, initially discovered as a regulator of food intake and energy expenditure, is emerging as a pleiotropic cytokine involved in immune and inflammatory responses. In Chapter 5, we focused on the role of leptin as proinflammatory mediator in the local inflammatory response in COPD. Leptin was detectable in induced sputum of male COPD patients. Sputum leptin showed a strong positive correlation with sputum levels of CRP and TNF- α , whereas leptin was inversely related to sTNF-receptors in sputum. These correlations may indicate that leptin is involved in the local inflammatory response in COPD.

In Chapter 6, we paid attention to on another aspect of the local inflammatory response, that is the proteinase-anti-proteinase imbalance in COPD. MMPs are suggested to play a critical role in the development and progression of COPD, but quantitative data on MMP activity in airways are lacking. Using specific immunocapture assays that allow determination of both active and potentially activatable (total) MMPs, we compared activity of MMP-1, MMP-8, and MMP-9 in sputum of COPD patients and healthy smokers. COPD patients appeared to have increased

sputum activity of MMP-8 and MMP-9, whereas MMP-1 activity was not increased. MMP-8 and MMP-9 (active and total) were strongly correlated, and were related to the absolute number of neutrophils in both study groups. In addition, an inverse correlation between MMP-8 and MMP-9 activity in sputum and airflow obstruction (FEV₁) was found in the combined study groups. Immunohistochemistry demonstrated strong positive staining for MMP-9 in both alveolar macrophages and neutrophils, whereas specific staining for MMP-8 was exclusively observed in neutrophils. The presence of increased MMP-8 and MMP-9 activity in the airways of COPD patients supports the concept of an impaired proteinase-anti-proteinase imbalance in COPD.

THE EFFECTS OF ACUTE AND CHRONIC LPS EXPOSURE TO THE LUNG IN MICE

Lipopolysaccharide (LPS) is ubiquitously present as contaminant on airborne particles, including air pollution and organic dusts. Chronic exposure to significant levels of LPS was reported to be associated with the development and/or progression of many types of lung diseases, including COPD. Recently, bioactive LPS was demonstrated to be present at high levels in cigarette smoke. Since long-term cigarette smoking is the major environmental factor that predisposes patients to COPD, LPS present in cigarette smoke may be a candidate to trigger smoking-induced accumulation of macrophages, lymphocytes, and neutrophils in respiratory bronchioles, alveolar ducts, and alveoli in susceptible cigarette smokers. Therefore, we performed animal studies to investigate the effects of acute and long-term LPS exposure to the lung *in vivo* in more detail.

To study the effects of acute LPS on inflammation and tissue injury, we made use of a well-characterized murine model. Single intratracheal instillation of 5 μ g LPS resulted in a rapid but transient inflammatory response, characterized by elevated TNF- α expression and a profound infiltration of neutrophils from the pulmonary vascular bed into the respiratory air spaces (Chapter 7). In addition, local LPS exposure appeared to result in early apoptosis of bronchial epithelial cells independent of infiltrating neutrophils and TNF- α . This indicates that exposure of the lung to LPS not only induces pulmonary inflammation, but may also be directly involved in rapid airway injury.

Expression of acute-phase reactants (APRs) at the site of LPS exposure may enhance the early innate immune response to injury and infection. Therefore, the effects of local LPS exposure on both pulmonary and hepatic expression of four major acute-phase proteins – SAP, AGP, LBP, and α_1 -AT – were assessed (Chapter 8). Local LPS exposure in mice resulted in a rapid increase of mRNA expression for three major APRs (α_1 -AT, AGP, and LBP) in the lung. In addition, exposure to LPS in the pulmonary compartment resulted in elevated levels of AGP, LBP, and SAP in the circulation, which was consistent with the APR expression pattern in the liver. Furthermore, the increased plasma IL-6 levels after local LPS exposure suggest that IL-6 acts as mediator between the pulmonary and hepatic system. As local

production of APRs results in higher concentrations in the intra-alveolar compartment and shortens the time path, pulmonary expression of APRs may be more efficient than extravasation of serum APRs to control the early phase of innate immunity to injury and infection.

In Chapter 9, the inflammatory and pathological effects of long-term LPS exposure to the lung were investigated in detail. To this end, a novel murine model in which mice were exposed to repeated intratracheal instillation of *E.coli* LPS was developed. Long-term LPS exposure was shown to result in chronic inflammation, characterized by peribronchial and perivascular lymphocytic aggregates (CD4+, CD8+, and CD19+), parenchymal accumulation of macrophages and CD8+ T cells, and altered cytokine expression (TNF- α , IL-18 and IFN- γ). The absence of neutrophils indicates that neutrophil infiltration is rather a transient process triggered by each LPS exposure instead of being a chronic factor. In addition, long-term LPS exposure resulted in significant airway and alveolar alterations such as increased mucus cell metaplasia in larger airways, thickening of the airway smooth muscle layer, and irreversible alveolar enlargement without proliferation of alveolar epithelial cells. Interestingly, the observed inflammatory and pathological changes in mice exposed to repeated local LPS exposure mimic important features observed in human subjects with chronic pulmonary inflammatory disorders, especially COPD. Therefore, this murine model could be applicable to dissect the role of inflammation in the pathogenesis of these disease conditions.

Chapter 10 comprises the general discussion of the thesis and gives implications for future investigations.

CHAPTER 12

Nederlandse samenvatting

LONGEN

De longen zijn verantwoordelijk voor de zuurstofvoorziening in ons lichaam. Het goed functioneren van de longen is dus van levensbelang. De longen bestaan uit luchtwegen omringd door longblaasjes. De luchtwegen beginnen in de luchtpijp, en eindigen na een groot aantal vertakkingen in achtereenvolgens grote, middelgrote en kleine luchtwegen, uiteindelijk in de longblaasjes. De gasuitwisseling (van zuurstof en kooldioxide) vindt plaats tussen de wanden van de kleinste luchtwegen en longblaasjes, en hele kleine bloedvaatjes die dit weefsel omgeven (Figuur 1).

Figuur 1. Schematische weergave van (A) het lagere ademhalingskanaal met de luchtpijp, de grote luchtwegen en de kleinere luchtwegen; (B) het gaswisselingsweefsel met de kleinere luchtwegen en de longblaasjes (Uit: *Netter Atlas of Human Anatomy*).

COPD

“Chronic obstructive pulmonary disease”, ofwel COPD, is de engelstalige benaming voor chronisch obstructieve longziekten. COPD is een chronische aandoening van de longen, die zich meestal op latere leeftijd uit in een geleidelijk en grotendeels onomkeerbare verslechterende longfunctie. Patiënten met COPD hebben longemfyseem, chronische bronchitis of een combinatie van beide ziektebeelden. Longemfyseem wordt gekenmerkt door versnelde afbraak van het elastische steunweefsel in de longen (“de rek is uit de long”). Dit leidt tot een

beschadiging en uiteindelijk verlies van longblaasjes. Hierdoor kan de gasuitwisseling in de longen minder goed plaatsvinden. Patiënten met chronische bronchitis hebben een voortdurend terugkerende overmatige slijmproductie in de luchtwegen, die vaak gepaard gaat met luchtweginfecties. Dit slijm zorgt voor vernauwing van de luchtwegen (obstructie). Hierdoor wordt de ademhaling ernstig bemoeilijkt. Veel voorkomende klachten bij COPD patiënten zijn dan ook kortademigheid, sputum (slijm) productie, veelvuldig hoesten en/of een piepende ademhaling.

Roken wordt gezien als de belangrijkste risicofactor voor de ontwikkeling van COPD. 80-85% van de patiënten met COPD is dan ook jarenlang een (zware) roker (geweest). Een belangrijk gegeven is echter dat niet alle rokers, maar slechts 10-20% in de loop der jaren COPD ontwikkelt. Dit betekent dat ook (tot nu toe onbekende) genetische factoren een rol spelen bij de ontwikkeling van COPD. Behalve het stoppen met roken, zijn er tot op heden geen effectieve behandelingen voorhanden die het ontstaan en het verloop van de ziekte kunnen remmen.

Door de hoge morbiditeit (voorkomen van klachten) en de hoge mortaliteit (overlijden aan de ziekte) van de ziekte, heeft COPD enorme (financiële) gevolgen voor de samenleving. Veel van de patiënten moeten hun werk opgeven, er moeten hoge kosten gemaakt worden voor bezoeken aan artsen en therapie, voor ziekenhuisopnames, en voor thuisbehandeling met zuurstoftherapie. De sterfte ten gevolge van COPD neemt nog steeds toe. De verwachting is dat COPD door de toenemende vergrijzing en luchtvervuiling in 2020 wereldwijd de derde doodsoorzaak zal zijn, na longkanker en AIDS. De geschatte mortaliteit is dan 4,7 miljoen personen per jaar.

Beter inzicht in de mechanismen die de basis vormen van de ontwikkeling en voortgang van COPD is noodzakelijk om uiteindelijk te komen tot effectieve therapieën. Het is duidelijk geworden dat chronische ontstekingsprocessen in de longen – inclusief de luchtwegen, longblaasjes en bloedvaten – een belangrijke rol spelen bij de vermindering van de longfunctie en in de longklachten van patiënten met COPD. In de afgelopen jaren is ook ontdekt dat deze ontstekingsreacties zich niet beperken tot de longen (lokale ontsteking), maar ook aanwezig zijn in de bloedbaan (systemische ontsteking). De oorzaak van de ontstekingsreacties in de bloedbaan van COPD patiënten is echter nog steeds onbekend.

LIPOPOLYSACCHARIDE

Lipopolysaccharide (LPS) is een belangrijk bestanddeel van de celwand van zogenaamde Gram-negatieve bacteriën. LPS is aanwezig op stofdeeltjes die voorkomen in de lucht die we inademen, zoals luchtverontreiniging, huisstof en organisch stof. Zoals schematisch is weergegeven in Figuur 2, bestaat LPS uit een vetgedeelte (phospholipid) en een suikerstaart (polysaccharide) en juist het vetgedeelte heeft sterke ontstekingsbevorderende eigenschappen. Verschillende studies hebben een relatie aangetoond tussen langdurige blootstelling aan hoge

concentraties LPS op stofdeeltjes en het ontstaan van chronische longaandoeningen, zoals astma en COPD. Recent is ontdekt dat bioactief LPS ook aanwezig is in sigarettenrook. Aangezien roken de belangrijkste risicofactor is voor het krijgen van COPD, zou LPS een belangrijke factor kunnen zijn in de voortgang van ontstekingsprocessen in COPD. Tot nu toe is er weinig onderzoek gedaan naar de effecten van chronische LPS blootstelling op ontstekingsprocessen in de longen.

Figuur 2. Schematische weergave van lipopolysaccharide (LPS) met het vetgedeelte (phospholipid) en de suikerstaart (polysaccharide) (Uit: Alexander C en Rietschel ET. *Bacterial lipopolysaccharides and innate immunity. J Endotoxin Res* 2001;7:167-202).

DOEL VAN HET ONDERZOEK

Het doel van het onderzoek is om meer inzicht te krijgen in de ontstekingsprocessen, zoals die voorkomen bij patiënten met COPD. Hiertoe hebben wij onderzoek gedaan naar:

- De ontstekingsprocessen in de luchtwegen en de bloedbaan bij COPD patiënten en de relatie tussen deze processen;
- De effecten van acute of langdurige blootstelling aan LPS op de longen door gebruik te maken van een dierexperimenteel model.

LOKALE EN SYSTEMSICH ONTSTEKINGSREACTIES IN COPD

Goed inzicht in de ontstekingsreacties in de longen en de bloedbaan is van belang voor toekomstige behandeling én preventie van COPD. Tot nu toe is onze kennis over chronische ontstekingsprocessen in COPD beperkt en slechts gebaseerd op enkele studies in meestal ernstige COPD patiënten. In Hoofdstuk 3 t/m 6 hebben we (lokale) ontstekingprocessen in de luchtwegen en (systemische) ontstekingprocessen in de bloedbaan van patiënten met COPD bestudeerd en dit vergeleken met gezonde rokende vrijwilligers. Aangezien het niet mogelijk is om

zomaar een stukje longweefsel weg te nemen zonder medische reden, hebben we gebruik gemaakt van opgewekt sputum (slijm) om ontstekingscellen en opgeloste stoffen (mediatoren) in de luchtwegen te bestuderen. Dit sputum wordt op een eenvoudige en veilige manier opgewekt (losgeweekt) door het inademen van een nevel van zout water. Daarna wordt het sputum opgehoest. Ook is een buisje bloed afgenomen om de ontstekingsprocessen in de bloedbaan te kunnen meten.

In Hoofdstuk 3 hebben we verschillende typen ontstekingscellen en ontstekingsmediatoren (IL-8, TNF- α en oplosbare receptoren voor TNF- α) bestudeerd in opgewekt sputum en bloedplasma. COPD patiënten bleken meer neutrofielen (een bepaalde ontstekingscel) en ontstekingsmediatoren in hun sputum te hebben vergeleken met gezonde rokers. Een andere belangrijke bevinding was dat er een sterk verband was tussen de mate van lokale ontsteking en de longfunctie. Individuen met meer ontstekingscellen en/of ontstekingsmediatoren in hun sputum hadden namelijk een slechtere longfunctie. Ook in de bloedbaan van COPD patiënten werden meer ontstekingsmediatoren gevonden dan bij gezonde rokers, maar dit was niet gerelateerd aan de longfunctie. Daarna werden de waarden van de ontstekingsmediatoren vergeleken tussen sputum en bloed, maar het bleek dat er geen verband bestond tussen de mate van lokale en systemische ontsteking. Deze resultaten suggereren dat de systemische ontsteking niet het gevolg is van lekkage van mediators vanuit de long.

In Hoofdstuk 4 werd een andere groep van mediators bestudeerd in opgewekt sputum en bloedplasma, namelijk acute-fase eiwitten (AGP, CRP en LBP). De precieze functies van de verschillende acute-fase eiwitten is nog onduidelijk, maar ze spelen zeker een belangrijke rol bij de verdediging van het lichaam tijdens infecties en/of ontsteking. Concentraties van AGP en LBP (maar niet CRP) waren duidelijk verhoogd in sputum van COPD patiënten. Net als de ontstekingsmediatoren (Hoofdstuk 3) waren hoge concentraties van AGP en LBP in sputum gerelateerd aan een slechte longfunctie. In bloedplasma was alleen LBP verhoogd in COPD patiënten, dit was juist gerelateerd aan een goede longfunctie. Ook nu vonden we geen verband tussen de lokale en systemische ontstekingsprocessen in COPD. Deze resultaten suggereren dat de acute-fase eiwitten gemeten in sputum lokaal in de long gemaakt worden, en bijdragen aan de lokale verdediging van de long.

In Hoofdstuk 5 hebben we leptine bestudeerd. Leptine is een hormoon dat een belangrijke rol speelt bij de regulatie van de energiebalans in ons lichaam. Recent is ontdekt dat leptine ook betrokken is bij ontstekingsprocessen. Daarom hebben we onderzocht of leptine voorkomt in sputum van COPD patiënten en mogelijk kan dienen als een marker van ontstekingsprocessen in de long. Leptine bleek inderdaad aanwezig te zijn in sputum van COPD patiënten en duidelijke relaties werden gevonden met concentraties van CRP en TNF- α in sputum. Deze resultaten suggereren dat leptine betrokken is bij de lokale ontstekingsprocessen in de long van COPD patiënten.

In Hoofdstuk 6 werd aandacht besteed aan een ander aspect van de lokale ontsteking, namelijk activiteit van matrix metalloproteïnases (MMP's) in sputum.

MMP's zijn enzymen die andere eiwitten kunnen afbreken en sterke activiteit van MMP's kan zo leiden tot beschadiging cq. afbraak van weefselstructuren, zoals longblaasjes. Recente studies laten zien dat concentraties van MMP's verhoogd zijn in de long (longspoelsel, sputum, longweefsel) van COPD patiënten, echter de activiteit van MMP's is tot nu toe niet gemeten. Door gebruik te maken van een nieuwe meetmethode hebben we de activiteit van verschillende MMP's (MMP-1, MMP-8 en MMP-9) in sputum kunnen meten. COPD patiënten bleken een verhoogde activiteit van MMP-8 en MMP-9 te hebben vergeleken met gezonde rokers. Verhoogde MMP activiteit was sterk gerelateerd aan een slechte longfunctie. Met behulp van antistoffen werd vervolgens gekeken welke ontstekingscellen aanwezig in opgewekt sputum deze MMP's maken. Het bleek dat MMP-8 alleen door neutrofielen gemaakt werd, terwijl zowel neutrofielen als macrofagen (een ander type ontstekingscel) het MMP-9 maken. Deze resultaten ondersteunen de hypothese dat MMP's een belangrijke rol spelen in het ontstaan en de voortgang van COPD.

EFFECTEN VAN ACUTE EN CHRONISCHE LPS BLOOTSTELLING OP DE LONG

Om de effecten van acute en chronische LPS blootstelling op de long te bestuderen, werden verschillende dierexperimentele studies uitgevoerd (Hoofdstuk 7 t/m 9). We hebben hiervoor gebruik gemaakt van een model, waarbij muizen via de luchtpijp (lokaal) 5 μ g LPS kregen toegediend.

In Hoofdstuk 7 hebben we gekeken naar acute ontstekingsreacties en acute schade aan het longweefsel veroorzaakt door eenmalige blootstelling aan LPS. Het bleek dat lokale LPS blootstelling resulteerde in een productie van ontstekingsmediatoren (TNF- α) en ophoping van neutrofielen in de longen. Er was echter ook directe schade door lokale LPS toediening, onafhankelijk van deze acute ontstekingsreactie: een deel van de bronchiale epitheelcellen (cellen die de binnenwand van de luchtwegen bekleden) bleek namelijk dood (in apoptose) te gaan. Deze studie toont aan dat blootstelling van longen aan LPS niet alleen leidt tot acute ontstekingsreacties, maar ook direct betrokken is bij beschadiging van de luchtwegen.

In Hoofdstuk 8 werd het effect van LPS blootstelling in de long op de productie van vier acute-fase eiwitten – SAP, AGP, LBP en α_1 -AT – in de long, de lever en de bloedbaan bestudeerd. De lever is het orgaan wat normaal zorg draagt voor de productie van acute-fase eiwitten, die via de bloedbaan naar de rest van het lichaam, inclusief de longen, worden getransporteerd. Het bleek dat lokale blootstelling aan LPS leidde tot een snelle productie van acute-fase eiwitten in de long. Deze resultaten suggereren dat de lokaal gemaakte acute-fase eiwitten bijdragen aan een snelle adequate verdediging van de long tijdens infectie en/of ontsteking. Ook werden verhoogde waarden van acute-fase eiwitten in de bloedbaan gemeten. Het bleek dat tevens de lever geactiveerd werd tot productie van acute-fase eiwitten door LPS blootstelling in de long. Deze resultaten tonen aan dat blootstelling van longen aan LPS niet alleen lokale effecten heeft, maar ook

leidt tot activatie van de lever.

In Hoofdstuk 9 zijn de effecten van langdurige blootstelling aan LPS bestudeerd op ontstekingsprocessen en schade in de long. Hiertoe werd een nieuw muizenmodel ontwikkeld: muizen werden 2 keer per week blootgesteld aan 5 μ g LPS gedurende 12 weken. Het bleek dat deze langdurige lokale LPS blootstelling leidde tot een ontstekingspatroon, dat zelfs 8 weken na de laatste LPS blootstelling bleef bestaan. Daarom spreken we hier van een chronische ontstekingsproces. Verder bleek dat langdurige LPS blootstelling leidde tot chronische schade aan de long die lijkt op de longschade zoals die voorkomt bij patiënten met COPD: verhoogde aantallen slijmproducerende cellen, verdikking van de spierlaag rondom de luchtwegen en afbraak van de wand van longblaasjes. Dit nieuwe model lijkt geschikt om de relatie tussen de chronische ontstekingsprocessen en schade in de long te bestuderen.

CONCLUSIES

De belangrijkste conclusies uit de in dit proefschrift beschreven studies zijn:

1. De ontstekingsprocessen in de luchtwegen van COPD patiënten worden gekenmerkt door a) verhoogde aantallen neutrofielen, b) hogere concentraties ontstekingsmediatoren en acute-fase eiwitten, en c) verhoogde activiteit van MMP's in vergelijking met gezonde rokers;
2. COPD patiënten hebben een systemische ontsteking, die gekenmerkt wordt door hogere concentraties ontstekingsmediatoren en acute-fase eiwitten in vergelijking met gezonde rokers;
3. De mate van ontsteking in de longen (niet in de bloedbaan) is gerelateerd aan een slechtere longfunctie;
4. Er is geen verband gevonden tussen de lokale en systemische ontstekingsprocessen in COPD patiënten;
5. Acute LPS blootstelling in de longen van muizen leidt tot a) lokale ontsteking, b) directe longschade, c) lokale productie van acute-fase eiwitten en d) activatie van de lever;
6. Langdurige LPS blootstelling in de longen van muizen leidt tot chronische ontsteking en weefselschade gelijkend op COPD.

We hebben nog een lange weg te gaan om de ziekte COPD beter te begrijpen. Meer onderzoek naar chronische ontstekingsprocessen, zowel in de longen als in de bloedbaan, is nodig om aangrijpingspunten te vinden voor nieuwe behandelmethoden. Dierexperimentele studies kunnen hier een belangrijke bijdrage aan leveren. De huidige en toekomstige impact van deze ziekte voor de patiënt, de directe omgeving en de maatschappij vragen bovendien om een voortvarende aanpak van deze "silent killer".

Abbreviations

α_1 -AT	:	α_1 -antitrypsin
α -SMA	:	α -smooth muscle actin
AGP	:	α_1 -glycoprotein
APR	:	Acute-phase reactant
AT	:	Annealing temperature
BAL	:	Bronchoalveolar lavage
COPD	:	Chronic obstructive pulmonary disease
DTT	:	Dithiothreitol
dUTP	:	2'-deoxy-uridine-5'-triphosphate
ECM	:	Extracellular matrix
ELISA	:	Enzyme linked immunosorbent assay
FEV ₁	:	Forced expiratory volume in 1 second
H&E	:	Hematoxylin & eosin
IFN- γ	:	Interferon-gamma
IL	:	Interleukin
IP	:	Intraperitoneal
IT	:	Intratracheal
LBP	:	LPS binding protein
L _M	:	Mean linear intercept
LM	:	ligation-mediated
LPS	:	Lipopolysaccharide
LT	:	Lymphotoxin
mAb	:	Monoclonal antibody
MMP	:	Matrix metalloproteinase
mRNA	:	Messenger RNA
MHC	:	Major histocompatibility complex
MPO	:	Myeloperoxidase
pAb	:	Polyclonal antibody
PAS	:	Periodic acid-Schiff
PCR	:	Polymerase chain reaction
RT	:	Reverse transcriptase
SAP	:	Serum amyloid P component
s	:	Soluble
SD	:	Standard deviation
SEM	:	Standard error of the mean
TNF- α	:	Tumor necrosis factor alpha
TNF-R55	:	TNF-receptor 55
TNF-R75	:	TNF-receptor 75
TUNEL	:	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling

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A handwritten signature in black ink. The name 'Juanita' is written in a cursive script. A large, sweeping underline starts from the end of the name and extends diagonally upwards and to the right, ending with a small dot.

List of publications

FULL PAPERS

Vernooy JH, Kucukaycan M, Jacobs JA, Chavannes NH, Buurman WA, Dentener MA, and Wouters EF. Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. *Am J Respir Crit Care Med* 2002;166:1218-1224.

Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA, and Wouters EFM. Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology. *Am J Respir Cell Mol Biol* 2002;26:152-159.

Vernooy JH, Dentener MA, van Suylen RJ, Buurman WA, and Wouters EF. Intratracheal instillation of lipopolysaccharide in mice induces apoptosis in bronchial epithelial cells: no role for tumor necrosis factor-alpha and infiltrating neutrophils. *Am J Respir Cell Mol Biol* 2001;24:569-576.

Dentener MA, Vreugdenhil AC, Hoet PH, Vernooy JH, Nieman FH, Heumann D, Janssen YM, Buurman WA, and Wouters EF. Production of the acute-phase protein lipopolysaccharide-binding protein by respiratory type II epithelial cells: implications for local defense to bacterial endotoxins. *Am J Respir Cell Mol Biol* 2000;23:146-153.

Stalman M, Koskamp A, Luderer R, Vernooy JHJ, Wind JC, Wullems GJ, and Croes AF. Regulation of anthraquinone biosynthesis in cell cultures of *Morinda citrifolia*. *J. Plant Physiol* 2003;In press.

Vernooy JH, Lindeman J, Jacobs JA, Hanemaaijer R, and Wouters EF. Increased activity of MMP-8 and MMP-9 in induced sputum from patients with COPD. *Am J Respir Crit Care Med* 2003;Submitted.

Vernooy JH, Dentener MA, Buurman WA, and Wouters EF. Local and systemic acute-phase response in COPD. *Eur Resp J* 2003;Submitted.

Vernooy JH, Reynaert N, Wolfs TGAM, De Vries B, Dentener MA, Buurman WA, and Wouters EF. Rapid expression of acute-phase reactants in lung after local LPS exposure in mice. *Infect Immun* 2003;Submitted.

Broekhuizen R, Vernooy JHJ, Schols AMWJ, Dentener MA, and Wouters EFM. Leptin as local inflammatory marker in COPD. *Eur Resp J* 2003;Submitted.

BOOK CONTRIBUTIONS

Vernooy JHJ, Dentener MA, Buurman WA, and E.F.M. W. 2002. Intratracheal instillation of lipopolysaccharide in mice results in increased hyaluronan deposition in the lung. *In* J.F. J.F. Kennedy, G.O. Phillips, P.A. Williams, and V.C. Hascall, editors. *HYALURONAN - (Bio)Chemical, Medical and Clinical Aspects*, ed. Woodhead, Cambridge.

Wouters EFM, Dentener MA, Vernooy JHJ, Pouw EM, Engelen MPKJ, and Creutzberg EC. 2003. AECOPD: a systemic disease? *In* N. Siafakis, N. Anthonisen, and D. Georgopoulos, editors. *AE-COPD*, ed. Marcel Dekker Inc., New York.

ABSTRACTS (SELECTION)

Vernooy JHJ, Küçükaycan M, Dentener MA, Buurman WA, and Wouters EFM. Local acute-phase response in subjects with stable COPD. *Am. J. Resp. Crit. Care Med.* 2002;165:A603.

Vernooy JHJ, Reynaert N, Dentener MA, Buurman WA, and Wouters EFM. Intratracheal instillation of lipopolysaccharide in mice results in increased hyaluronan deposition in the lung. *Am. J. Resp. Crit. Care Med.* 2002;165:A757.

Vernooy JHJ, Küçükaycan M, Dentener MA, Buurman WA, and Wouters EFM. Differences in soluble TNF-receptors 55 and 75 in induced sputum from patients with COPD and healthy controls. *Am. J. Resp. Crit. Care Med.* 2002;165:A824.

Vernooy JHJ, Dentener MA, Koerts-De Lang E, Van Suylen RJ, Buurman WA, and Wouters EFM. Chronic pulmonary inflammation induced by repeated lipopolysaccharide instillation in mice results in coexistent emphysema and bronchitis. *Am. J. Resp. Crit. Care Med.* 2000;161:A181.

Vernooy JHJ, Dentener MA, Van Suylen RJ, Buurman WA, and Wouters EFM. Intratracheal instillation of lipopolysaccharide induces apoptosis in bronchial epithelial cells. *Am. J. Resp. Crit. Care Med.* 1999;159:A697.

Vernooy JHJ, Lindeman J, Jacobs JA, Hanemaaijer R, and Wouters EFM. Increased activity of MMP-8 and MMP-9 in induced sputum from patients with mild-to-moderate COPD. *Am. J. Resp. Crit. Care Med.* 2003; In press.

Broekhuizen R, Vernooy JHJ, Schols AMWJ, Dentener MA, and Wouters EFM. The interaction between leptin and inflammatory markers in induced sputum in patients with moderate COPD. *Eur Resp J* 2002;20:404S.

Möller GM, Vernooy JHJ, and Wouters EFM. Increased expression of Granzyme A

in patients with COPD. *Am. J. Resp. Crit. Care Med.* 2002;165:A603.

Hageman GJ, Larik I, Vernooij JHJ, Dentener MA, Bast A, and Wouters EFM. Exposure of lung epithelial cells to reactive oxygen species induces PARP-1 activation in vitro and in vivo. *Am. J. Resp. Crit. Care Med.* 2002;165:A602.

Dentener MA, Vreugdenhil ACE, Vernooij JH, Janssen YMW, Buurman WA, and Wouters EFM. Production of the acute-phase protein LPS binding protein (LBP) by alveolar type II epithelial cells. *Am. J. Resp. Crit. Care Med.* 1999;159:A179.

154

Curriculum vitae

Juanita Vernooy was born on November 29th, 1973 in Heerlen, The Netherlands. She attended secondary school (Gymnasium-ß) at the "Sint-Janscollege" in Hoensbroek from 1986 till 1992. In the same year, she started the study Biology at the Faculty of Science, Mathematics and Computing Science, University of Nijmegen, and specialized in Physiology and Biochemistry. She graduated in 1997 on a research project performed at the Department of Molecular Plant Physiology, University of Nijmegen in close collaboration with the Department of Plant Physiology, Wageningen University, in which production of secondary metabolites was studied in transient and continuous cultures of *Morinda citrifolia* root cells.

In 1997 she started with her PhD project at the Department of Respiratory Medicine (formerly known as Pulmonology), Maastricht University, under supervision of Prof. dr. E.F.M. Wouters. The research presented in this thesis was financed by Glaxo Wellcome, The Netherlands, and was performed in close collaboration with the Department of General Surgery, Maastricht University, under supervision of Prof. dr. W.A. Buurman. Nowadays, she is working as Post-doc at the Department of Respiratory Medicine, Maastricht University.